THE EFFECT OF INSULIN ON FETAL AND NEONATAL AMINO ACID METABOLISM

by

Merete M. Giles

Doctor of Philosophy,
Department of Child Life and Health,
University of Edinburgh.

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e of Thesis THE EFFECT OF INSULIN ON FETAL AND NEONATAL AMINO ACID METABOLISM

Two aspects of perinatal amino acid metabolism were studied:-

1. The influence of fetal insulin infusions on the placental transfer of amino acids, L phenylalanine leucine and lysine was investigated in sheep of 115 - 127 days gestation.

   It was confirmed that ^{14}C labelled amino acids were transferred readily from the mother to fetus during a continuous maternal infusion, plasma water activity reaching a plateau in 1 - 2 hours.

   However, most of the plasma activity was contained in the albumin fraction of the proteins, 89% in the mother and 78% in the fetus.

   Comparing ^{3}H and ^{14}C labelled amino acids, total plasma activity was lower and plasma water activity higher when ^{3}H labelled phenylalanine was infused.

   Insulin infusions were started after 4 hours continuous labelled amino acid infusion when steady state transfer conditions had been achieved. Insulin concentrations increased 8 - 56 fold but placental amino acid transfer as judged by changes in amino acid SA was not significantly changed. Fetal plasma water label remained constant whilst amino acid concentrations decreased, suggesting that increased net protein synthesis occurred, probably by decreased fetal protein catabolism.

   Fetal phenylalanine flux (prior to insulin infusion) was calculated at 231 and 137 umol/kg/hr, and leucine and lysine flux at 217 umol/kg/hr and 318 umol/kg/hr respectively.

2. The influence of insulin on plasma amino acid concentrations during intravenous feeding in preterm infants.

   During insulin infusions of 0.2 iU/kg/hr, the plasma amino acids decreased but the rapidity duration and extent of the response varied; such infusions may, therefore, be of limited value in assisting the tissues to take up and retain amino acids.

I hereby declare that this thesis has been written by myself and that except where acknowledged, the work was my own.

Merete M. Giles

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THE EFFECT OF INSULIN ON FETAL AND NEONATAL AMINO ACID METABOLISM

Optimal fetal growth, resulting in the birth of a well developed term infant is only possible if the intrauterine environment is capable of satisfying all fetal requirements. Amongst the most important of these requirements is the supply of nutrients, of which amino acids are of obvious importance for protein synthesis. The availability of amino acids is dependent both on maternal nutrition and health and the normal development and function of the placenta. The utilisation of nutrient by the fetus *in utero* is determined, to a large extent, by the hormonal and genetic status of the developing fetus itself, although maternal and placental metabolism can modify that of the fetus. Postnatally genetic and hormonal control passes entirely to the infant, who is no longer subject to either adverse or protective influences of the intrauterine environment - an example of adverse conditions being those experienced by an infant of a phenylketonuric or diabetic mother and an example of *in utero* protection being that of an infant with Maple Syrup Urine disease.

In this study unanaesthetised, unstressed sheep in the last trimester of pregnancy have been used to study placental transfer of the L-amino acids phenylalanine, leucine and lysine, and their subsequent utilisation by the fetus.

The influence of fetal insulin concentration on placental transfer and fetal amino acid metabolism in the sheep is reported, together with some observations on the effects of insulin on the post natal metabolism of intravenously fed preterm human infants.

Preterm, but appropriate weight for gestation infants have grown and developed normally *in utero* until delivery. After birth the paediatric objective for these infants is to promote growth and development at a rate similar to that which would have been subsequently achieved *in utero*. However, immaturity of organ function and lack of nutritional reserves handicap the preterm infant whose nutritional requirements per Kg body weight exceed those of the term infant. In order to achieve optimal nutrition in the preterm infant the normal nutritional supply and demand *in utero* must be known and the changed and possibly increased demands of extra-uterine life studied. In this thesis, placental transfer and fetal metabolism of amino acids are investigated in an attempt to improve understanding of fetal nutrition. Particular attention has been given to the role of insulin in fetal growth and its possible use in reversing catabolism and promoting anabolism in the sick preterm infant.

Mother and fetus are both ultimately dependent for amino acids on maternal dietary protein and its efficient absorption and utilisation. The fetus is additionally dependent on the transport of available nutrients across the placenta and any impairment to the nutrient supply due to either maternal dietary deprivation or placental insufficiency, can reduce fetal growth.

Growth depends not only on a supply of amino acids but also on fetal utilisation of these in peptide and protein synthesis, the two phenomena being inseparably linked. In the steady state rates of protein synthesis and catabolism are equal but in the developing fetus rapid growth must result in net synthesis exceeding net catabolism. These rates may be measured using radioactive (and more recently with ^{13}C and ^{15}N labelled[1]) amino acids but this

method of approach is obviously only possible in animal species, and the majority of studies have been "acute" experiments in anaesthetised animals.

Only recently have "chronic" animal studies been used in which catheterisation of fetal vessels permitted physiological and biochemical observations in the conscious animal.

Even so, the influence on these observations of relatively minor stresses to which the mother may be subjected and the length of time required to regain a steady physiological state following catheterisation procedures has not been recognised. Care was therefore taken to ensure that animals used in the experiments were in a near normal physiological condition.

Maternal placental and fetal hormones have been shown to influence fetal growth in a variety of animals[4,5].

In man, fetal pancreatic insulin has a major influence on fetal growth[6-9]. In this study the action of insulin on fetal metabolism was examined by infusing 5 sheep fetuses under steady state conditions. Changes in transfer and plasma clearance rates of labelled amino acid were measured to assess the response to insulin.

The rate of growth in the newborn as in the fetus must correlate with the rate of protein synthesis. In order to achieve anabolism and growth the dietary protein intake must exceed basal metabolic requirements. In the normal term infant receiving an adequate milk intake dietary protein is available for protein synthesis. Howeverinthe preterm or light for date (LFD) infant not only may dietary intake and intestinal absorption be diminished relative

to size, but obligatory energy expenditure may be increased, e.g. through increased heat and water losses. In addition, energy reserves of glycogen and fat are small. The preterm and LFD infant are, therefore, more likely to utilize dietary protein and body tissue (primarily muscle) protein in order to meet these energy requirements. When requirements cannot be met by enteral feeding, parenteral infusions of amino acids, fat, carbohydrate mineral solution and vitamins can reverse catabolism and promote protein synthesis and growth. Under very carefully controlled conditions it may be possible to further increase the protein synthetic rate by supplying exogenous insulin. Changes in neonatal plasma concentrations of amino acids, fat and glucose were monitored in infants receiving total parenteral nutrition to assess whether the infusion of insulin could promote anabolism.

LITERATURE BACKGROUND

i MATERNAL, FETAL AND NEONATAL AMINO ACID ENVIRONMENT

Amino acid concentrations of maternal and fetal fluids have been measured, and changes in these examined in relation to gestational age, pregnancy complications and maternal nutrition. Abnormal concentration relationships between mother and fetus could indicate either altered transfer rates across the placenta or altered fetal utilisation.

a. Maternal Amino Acid Environment

Both essential and non essential free amino acid concentrations decrease in maternal plasma during the first trimester of pregnancy and are maintained at this new lower concentration until pregnancy terminates, when non-pregnant values are rapidly regained.[10,11,12,13,14,15,16]

There is little doubt that endocrine changes, in addition to fetal demands, are responsible for this general reduction, probably through increasing cellular uptake into the uterus and its contents,[17,18,19,20] and increasing amino acid, urinary excretion.[12,18,21]

Additionally, Bjornesjo observed that although amino acid concentrations were decreased in plasma during pregnancy, red blood cell concentrations were increased. This he felt might also have been mediated through endocrine influences, (oestrogen, insulin) there being an increased uptake of amino acids from plasma.[" 22 "]

b. Fetal and Neonatal Plasma Amino Acid Environment

That amino acids are at a greater concentration in fetal than in maternal plasma water has been observed by many investigators.[13,14,15,16,23,24,25]

The ratio of umbilical venous (uv):maternal venous (mv) free amino acid concentrations in man is between 1:1 and 2:1 for

most amino acids, although changes in the ratio of individual amino acids occur as pregnancy progresses. Highest uv concentrations of plasma free amino acids have been observed during the second trimester of pregnancy.[13,25] The ratio in the sheep is higher than in man, while that of the guinea pig falls between the two.[26]

Small, but characteristic, changes in plasma composition occur during the first three days of neonatal life, most amino acids decreasing.[27,28]

c. Amniotic Fluid, Fetal and Neonatal Urine Composition

Like fetal plasma, free amino acid concentrations in amniotic and fetal urine decrease during the third trimester.[25] Fetal urinary amino acid concentrations are generally below those of fetal plasma. In mid pregnancy there is a linear correlation for ten amino acids between concentrations in amniotic fluid and fetal urine in man, but at term, when large volumes of amniotic fluid (total volume 1000 ml) are swallowed by the fetus (approximately 450 ml/day)[31,32] no correlation exists.[25] Correlations between mv (8) and umbilical arterial (7) concentrations however, are found.

At birth, there is a rapid increase in urinary amino acid concentration so that urine amino acid concentrations are higher than that of neonatal plasma.[25]

d. Disorders of the Amino Acid Environment

Deviation from the normal free plasma amino acid concentrations e.g. in phenylketonuria and pre-eclampsia may be associated with abnormal fetal growth, reflecting changes in either fetal metabolism or placental transfer.

Free amino acid concentrations in plasma of both maternal and fetal blood have been found to be increased in pregnancies of pre-eclamptic mothers with LFD (light for date) infants.[23,28,30] However, the uv:mv ratios were decreased, particularly those of branched-chain amino acids (BCAA) and urea. It is suggested that alterations in hormonal balance and maternal blood flow in these mothers reduces the placental uptake, and thereby transfer to the fetus of both amino acids and glucose.[23] This results in utilization of amino acids for gluconeogenesis. Thus the high fetal uv concentration of plasma free amino acids represents fetal starvation rather than protection of the fetus from adverse placental conditions. This is supported by the finding that although in plasma of phenylketonuric mothers the phenylalanine concentration was increased, the uv:mv ratio was relatively normal.[33] The fetal plasma free phenylalanine: tyrosine ratio however was 8.16:1 compared with 2.47:1 in normal pregnancies (see page 26).

Abnormal hormonal status in maternal diabetes has little effect on plasma amino acid concentrations, but significantly increases fetal growth rate, including that of the B islets of the fetal pancreas, resulting in an increased fetal insulin concentration (8.2 µl/ml compared with 53.5 µl/ml).[13,23,34,35]

Despite similar post natal diets, the plasma amino acids and protein of LFD and large infants may still show variations from those of normal infants seven days after birth.[23,28] Additionally, in the preterm infant, a limited dietary intake coupled with limited digestive ability, immature enzyme function and, often,

clinical illness (ideopathic respiratory distress syndrome, infection, biochemical and coagulation problems) may significantly alter the plasma amino acid profile from that of normal human milk fed term infants.

Significant alterations can also occur when parentral feeding is employed in preterm infants to increase nutrient intake. Many of the changes induced by parenteral feeding are governed by external factors e.g. amino acid solution used, rate of infusion and balance with other calorie sources,[36,37] but alterations can also be related to immaturity, and these changes must be considered when assessing the requirements for any form of nutrition.

Small carbohydrate and fat reserves, in addition to a low caloric intake may force the preterm infant to use amino acids for gluconeogenesis. Thus, the plasma amino acid profile might resemble that observed in the plasma of starved children and adults - with alanine, glutamine, glycine, histidine, serine, methionine, isoleucine and ornithine all being reduced.

Impaired gluconeogenesis resulting from enzyme immaturity might, however, produce the opposite effect and plasma alanine, aspartic acid, serine, glycine and methionine concentrations could increase. Immature enzyme function might also influence methionine and cystine, phenylalanine and tyrosine concentrations (see page 22), cystine, tyrosine, histidine and arginine being essential not only for preterm but also term infants.[36,38,39]

The use of insulin in these small preterm parenterally fed infants to promote protein synthesis would probably only be effective if there was a balanced supply of amino acids, dietary amino acid

imbalance having been shown to reduce growth rate.[40-53]

Both acute and chronic maternal under and/or malnutrition has been found to be associated with an increased incidence of intrauterine growth retardation,[54,55] with the infant being further disadvantaged by poor maternal lactation.[56,57,58] However, diet may not be the only factor responsible, hard manual work of the women in many of the studies causing an alteration in blood flow patterns and a decreased flow to the uterus and placenta.[57] Uv:mv ratios and concentrations of plasma free amino acids resembled those of LFD infants of pre-eclamptic mothers.[59,60,61] Hence, although different factors may be responsible for decreased placental transfer the fetus responds in a similar manner.

The ratio of plasma free glycine + serine + glutamine + taurine : leucine + isoleucine + valine + methionine concentrations has been used to assess dietary deficiency.[62,63] This showed that metabolism in the perinatal period was different in LFD infants, the ratio being increased on day one, irrespective of gestation, in contrast to that or normal infants which decreased. The increase was caused by depressed essential amino acid concentrations.

In man, anaesthesia during labour has little effect on maternal amino acid concentrations,[15] alanine and cystine falling slightly. However, these two amino acids have been found to be increased in Caesarean sections together with lysine and glutamic acids.[23] Surgery to implant fetal catheters, however, has a profound effect on amino acid concentrations.[64] These changes are discussed on page 205.

e. Abnormalities of Placental Structure

As the supply of fetal nutrients depends on the efficient functioning of the placenta, it might be thought that the placental lesions would result in a growth retarded infant. However, although Gruenwald (1963)[65] found lesions in placentae of 36-41 week gestational infants, the infants showed no detectable growth retardation. Only occasionally could fetal growth retardation be ascribed to either smaller placentae or gross lesions. The study did not preclude the possibility of retardation through minute occlusions of the placental villi.

Later, Aherne and Dunnill reported that the placental volume and chorionic villous surface area were reduced in hypertension and that the ratio of parenchyma:non parenchymal tissue was reduced in small for dates infants.[66]

Pearse and Sornson[67] examining free amino acid contents of normal and abnormal placentae found an increase in concentration as pregnancy progressed. Infarcted placentae had low concentrations and placenta of pre-eclamptic mothers high amino acid concentrations. The reduced blood flow and therefore amino acid supply in the former and increased maternal plasma amino acid values in the second could well produce these effects.

The importance of good maternal and placental circulation for satisfactory fetal growth was studied in pigs by Wootton et al[68] (1977). Significant positive correlations between placental blood flow and both placental and fetal weight, compared with correlations between placental and fetal weights highlighted the problems a reduction in blood circulation through vascular disease or circumstance such as

TABLE I

MEAN PLASMA AMINO ACID CONCENTRATIONS IN PREGNANT SHEEP+

	Maternal		Fetal	
	Mean ±	SD	Mean ±	SD
Threonine	52	26	217	84
Valine	114	43	331	82
Methionine	14	5	25	9
Isoleucine	79	15	92	22
Leucine	67	26	106	34
Tyrosine	38	11	89	31
Phenylalanine	35	9	88	22
Lysine	131	37	113	36
Histidine	37	12	63	19
Serine	95	29	881	327
Glutamic Acid	125	36	135	50
Glutamine	208	77	355	185
Glycine	826	195	856	432
Alanine	124	32	271	117
Citruline	187	77	137	50
Ornithine	71	36	107	49
Arginine	145	46	125	57
Taurine	84	60	141	114
1 - Methylhistidine	58	14	130	42
3 - Methylhistidine	38	14	98	44
Asparagine	36	14	67	42
TAA	2565	382	4430	1055
EAA	568	159	1125	249
NAA	1378	260	2498	839

TAA = Total Amino Acids

EAA = Essential Amino Acids

NAA = Non-Essential Amino Acids

+ From Slater and Mellor (1979)[70]

hard physical work might cause. The relationship between blood flow and amino acid placental transfer is discussed in section ii, page 16.

f. The Relationship between Maternal and Fetal Amino Acid Environments in Animals

Animal studies have produced similar results to those reported in the human. Reports of free amino acid concentrations in the ewe show glycine, threonine, serine and alanine to be higher than in man and methyl histidines to be present.[69] Typical concentrations of leucine, lysine and phenylalanine, the three L amino acids employed in these experiments being 67 ± 26, 131 ± 37 and 35 ± 9 umol/l respectively in maternal plasma of sheep maintained under identical conditions to the sheep used in the experiments,[70] and 106 ± 34, 113 ± 36 and 88 ± 22 umol/l in fetal plasma.

Free amino acid concentrations for all amino acids are shown in Table I.

Animal experiments have enabled the effects of nutrition in pregnancy to be studied more fully. The relative proportion of infused amino acids being utilised for protein synthesis vary with altered energy and nitrogen intakes.[71] Reilly and Ford (1971) and Slater and Mellor (1972)[72] found the plane of nutrition in sheep influenced the metabolic rate of both carbohydrates and amino acids. A low plane of nutrition increased the utilisation of amino acids in gluconeogenesis and total plasma protein significantly decreased and although total amino acids remained unchanged the non-essential: essential amino acid ratio in both maternal and fetal plasma had

increased by 20 weeks gestation.[72] The fetal:maternal ratios of amino acids were similar on both high and low nutritional planes suggesting that the fetus took a higher percentage of the available amino acids of the ewes on the low plane of nutrition.

Pregnancy increased the flux of amino acids, but not glucose.[73,74] However, only 3% of amino acids and 40% of glucose were oxidised to carbon dioxide compared to 62% of amino acids and 65% of glucose in the non-pregnant state. Setchell _et al_ found that both fructose and glucose are converted to glycogen and carbon dioxide in the fetus[75] but together only accounted for a small fraction of total carbon dioxide from uterus and contents. Anand (1979)[76] using well fed ewes, found all the fetal glucose to be from the mother, no glucose being formed by the fetus.

Animal models have been employed in the study of intrauterine growth retardation by compromising the nutrient supply to the fetus through either ligation of the uterine vessels or maternal diet restriction. The effect of this may vary with the time of gestation employed - late fetal growth being more sensitive to restriction.[77,78,79] Offspring of the two forms, although physically alike, may also differ metabolically,[80] therefore care must be taken in assessing data from these models.

ii PLACENTAL TRANSFER

The rate and degree of transport of amino acids across the placental barrier is influenced by the structure of the individual amino acids, blood flow and amino acid concentrations and the metabolic requirements of the fetus, placenta and to a lesser extent the mother.

Transport of amino acids across the placenta to the fetus, which takes place against a concentration gradient, is believed to be achieved by the following mechanism:[26, 75, 76, 77, 78, 79, 80, 81]

The first phase is active uptake of amino acids from the maternal plasma by the syncytiotrophoblast. These amino acids are then concentrated in placental parenchyma, resulting in placental free amino acid concentrations above those of both maternal and fetal plasma. Subsequent transfer to the fetus is therefore by diffusion down a concentration gradient, the fetus itself providing a genetic "pull" by rapid utilization of amino acids in protein synthesis.

a. Amino Acid Structural and Transport Groups

Transport of amino acids across the placenta shares characteristics with other tissues such as intestine[88] and brain.[89] Four groups of amino acids have been recognised:

a) neutral straight chain amino acids - alanine, glycine and serine

b) neutral branched chain amino acids - leucine, isoleucine and valine

c) acidic amino acids - glutamic and aspartic

d) basic amino acids - lysine, arginine and histidine.

On column chromatography phenylalanine, tyrosine and tryptophan elute between the neutral branched chain and basic amino acids whilst methionine, cystine and proline elute amongst the neutral amino acids. Further classification may be made according to their transport characteristics.[90,91,92,93,94] Neutral straight chain amino acids are actively transferred by an "A" (alanine) preferring system whilst neutral branched chain amino acids are exchanged across membranes by an "L" (leucine) preferring system,[26,82] this system being the fastest, the greater solubility of branched chain amino acids probably aiding their transfer across membranes.[92] These "A" and "L" systems and a basic amino acid transport system are energy, oxygen and sodium dependent.[95] Energy can be provided by anaerobic processes,[96] particularly in the fetus which is more resistant to hypoxia.[97] Phenylalanine, methionine and proline probably utilize both the "A" and "L" preferring systems. Acidic amino acids however, are not generally transferred intact, but are formed in the placenta by transamination of citric acid cycle by products resulting from placental metabolism.[98,99,100,101]

The transfer systems are stereospecific, L isomers being selectively transferred[102,103,104] and there is competition between amino acids within a group. Considerable overlap between transport groups has also been noted. A further late developing transport system for glycine and imino acids has been described in the neonatal kidney.[105-110] Neutral straight chain and acidic amino acids are metabolically the most labile, whilst neutral branched chain and basic amino acids are essential and, in addition to general metabolism, are utilized extensively in protein synthesis.

b. Transport of Amino Acids

1) Uptake and Transfer

Placental uptake of amino acids has been studied both *in vitro* and *in vivo*. Active uptake of amino acids is principally a function of the maternal aspect of the placenta. Microvesicles formed from the plasma membrane of the human placenta have a greater affinity (3 fold) for amino acid uptake in comparison with microvesicles formed from the intracellular membranes of the trophoblast[87], although the latter have a greater overall capacity (36 times) thus permitting concentration of amino acids.

Investigation of amino acid uptake into placental tissue slices by Schneider and Dancis[111] showed that the intracellular:extracellular ratio achieved was determined as much by the efflux rate as the rate of uptake. Acidic amino acids were rapidly taken up, producing a high ratio whilst basic amino acids accumulated more slowly because of efficient efflux. Neutral amino acid behaviour was similar to that of basic amino acids but no plateau was attained. Leucine was unusual in that a very rapid efflux rate resulted in a ratio below one. This could have been a result of the tissue preparation as previously it had been suggested that the physical structure of the placenta may be of importance, transport being "enhanced" in an intact human villus compared with that in slices[112].

The slow but progressive development of high intracellular amino acid gradients in placental tissue during *in vitro* experiments because of ineffectual efflux resembles that of immature tissue, for although mature tissue has a more rapid uptake efficient efflux rapidly results in a plateau concentration. Thus, although species (e.g. guinea pig and man) and individual amino acid (e.g. valine) differences have been noted[113], fetal tissues generally have a higher concentration of amino acids than those of adults.

Placental tissue also resembles fetal tissue in that it continued to function under anaerobic conditions, AIB uptake being only slightly diminished on replacing oxygen with nitrogen. However, addition of DNP to the medium resulted in a 40% reduction, and the addition of arsenate completely inhibited AIB uptake.[96,112] This contrasts with *in vivo* findings; transfer of amino acids across the placenta of guinea pigs infused in situ were little affected by maternal hypoxia DNP or KCN until after maternal function was impaired.[84]

Observations on amino acid uptake in placental tissue slices are in agreement with values calculated from the free amino acid concentrations in the trophoblast and a low maternal vein:trophoblast ratio were found for acidic amino acids (ratio 1:60 for glutamate). Neutral straight chain amino acids were intermediate in both concentration and ratio (1:10 for alanine), the concentration in the trophoblast of neutral branched chain and other essential amino acids being lower than acidic amino acids, thus reducing the gradient between maternal plasma and trophoblast (1:5-9).

This suggests that essential amino acids are readily transferred but that the acidic amino acids are concentrated in the placenta, a large percentage of these acidic amino acids probably being a product of placental metabolism rather than the previously suggested combined uptake from the uterine and umbilical plasmas.

Further evidence for this selective transfer was provided by the placental transfer rates of amino acids in the sheep during maternal loading with neutral straight and branched chain amino acids.[82,83] Whilst only a small increase in fetal plasma amino acid concentration was found in the former group, indicating only a slow transfer, both the free amino acid concentration and the umbilical plasma arterial:venous

difference increased in the latter, suggesting both rapid transfer and increased uptake by fetal tissues. In rats maternal loading with phenylalanine and tryptophan resulted in a lag phase before[115] fetal plasma amino acid concentrations increased. This was ascribed to the concentrating of amino acids within the placenta. Similarly in the previous experiment the attenuated and extended fetal concentration curve compared with that of the mother could well result from accumulation of amino acids within the placenta.

Species differences in the rate of transfer of some amino acids are apparent.[87] Basic and neutral straight chain amino acids are transferred relatively slowly across the sheep placenta, but more readily across that of the monkey. Essential amino acids however behave similarly in sheep, guinea pig and monkey. Cystine is not actively transferred in most species.[116] Larson *et al*[117] and Matthews and Laster[118] reported that inhibition of transport by the amino acid itself only occurs at very high (1 mm) unphysiological concentrations, and although effects may be additive these concentrations were not reached in these loading experiments.

2) Arterial-Venous Differences and Blood Flow

Measurement of uterine arterial-venous differences together with uterine blood flow enables direct assessment of uterine uptake, the majority of which is accounted for by the placenta and fetus, only 5% of the blood flow passing through the myometrium.[87] Holzman *et al*[98] found that in unstressed sheep there was a significant uptake of neutral and basic amino acids into the pregnant uterus (65% and 20% respectively of the total), and uptake correlated well with the uterine free amino acid arterial concentration, the only exception being glycine which is at a high concentration in sheep plasma.

Acidic amino acids, however, were not effectively removed, there being little difference in the uterine venous and arterial concentrations. Glutamic acid crosses the placenta only slowly or is not transferred.[119] During the studies of uterine and umbilical arterial-venous differences no significant transfer occurred, in agreement with previous findings in the rat, but Schneider,[103] using the tissue slice preparation found 49% of the glutamic acid presented to the placenta was taken up. However, only 10% of this was transferred, this being in comparison with a 75% transfer of leucine although only 16% of this amino acid was taken up. In contrast to the slow insignificant transfer of glutamic acid, glutamine is efficiently transferred, for although placental amination of glutamate accounts for a proportion, fetal uptake was found to be in excess of the combined placental uptake of glutamate from fetal and maternal plasma. This is also true of aspartic acid and asparagine.[98-101]

Perfusion of guinea pig placenta in situ has shown maternal blood flow to have a significant influence on amino acid transfer, a decreased uterine flow reducing the total amino nitrogen transfer.[84,120] A 30% reduction in blood flow not only reduced both glucose and amino nitrogen placental transfer, but neutral branched chain and basic amino acid transfer was reduced to a greater extent than that of acidic and neutral straight chain amino acids. Thus poor placental circulation could create an imbalanced as well as reduced supply of amino acids to the fetus.

Unlike placental uptake from maternal plasma, blood flow has only a small influence on the release of amino acids to the fetus.[84] Umbilical arterial concentration is a more important factor in determining transfer, an inverse relationship between arterial concentration and transfer having been documented by Prenton and Young[83] in guinea pig and sheep.[26,82]

Although fetal plasma concentrations of free amino acids are greater than those of maternal plasma, they are below those of the trophoblast, the trophoblast:fetal plasma amino acid ratio being between 1.5 and 2.7:1. Transfer to the fetus is therefore by diffusion. Supporting evidence for this comes from closed circuit perfusion of guinea pig placenta in situ[114], the perfusate amino acid concentration increasing until it approximates that of the placenta, when transfer ceased or was reversed. Relative concentrations of individual amino acids in fetal plasma however, are modified by fetal metabolism.

From calculations of uv-ua differences, the majority of amino acids are released into fetal plasma, but glutamine, glycine, ornithine and serine have been found at higher concentration in the arterial plasma, this occurring more frequently with increasing gestation[121,122]. Both Prenton and Young[121] and Hayashi et al[121] found that in man towards term alanine was the only non essential amino acid supplied in significant quantities. This differs from sheep in which glutamine is transferred more effectively.

Other studies by Velazquez et al[123] using umbilical arterial-venous differences in man showed that fetal tissues retained essential amino acids, with the surprising exceptions of leucine and valine, but that glutamic acid, glutamine, proline and alanine were released into fetal blood. These were subsequently removed by the placenta, although no further transfer to the mother was detected.

Theoretically net transfer from mother to fetus may be calculated from umbilical and uterine arterial-venous differences if the blood flow on both sides of the placenta is known. However, the relatively small differences in concentration (0-17 μ mol/l on the maternal side

and 12-28 u mol/l on the fetal side) compared with the standard error of the amino acid determinations (approximately 5%) makes accurate assessment difficult.[92] Holzman calculated the uterine uptake of nitrogen in the ewe to be 1.45g Kg^{-1} day^{-1} and that of carbon to be 3.9g Kg^{-1} day^{-1}. This compares with an umbilical uptake of 1.5g Kg^{-1} day^{-1} (fetal weight) of nitrogen and 3.9g Kg^{-1} day^{-1} of carbon.[124] However, as the placenta excretes ammonia and urea (formed in the fetus) into the uterine circulation, but has no urea synthesizing capacity, theoretical calculation of fetal uptake based on a uterine uptake of 1.45g Kg^{-1} day^{-1} and ammonia and urea excretion of 0.18g Kg^{-1} day^{-1} and 0.36g Kg^{-1} day^{-1} respectively would be 1.3g Kg^{-1} day^{-1} i.e. 90% of the uterine uptake. The excess over the estimated requirement of 1g Kg^{-1} day^{-1} for fetal growth and urea production could be due to the analytical difficulties and assumptions made. Alternatively, there may be other forms or routes of nitrogen excretion.

It should also be remembered that nutrients may reach the fetus by routes other than the placenta. Both L and D isomers have been demonstrated to cross the chorioamniotic membrane in both directions,[125] the L transfer being rapid and both L and D isomer transfer being inhibited by 24 DNP.

iii FETAL AND NEONATAL AMINO ACID BALANCE

a. Free and Bound Amino Acid Relationships

No correlation exists between the uv-ua plasma concentration differences of individual amino acids and their concentration in intracellular fluid or protein. Thus extensive fetal metabolism of many of the amino acids supplied by placental transfer must occur. More acidic and neutral straight chain amino acids are synthesized de novo in fetal tissues than in animals on a postnatal diet. In addition to this increased demand for essential amino acids, the requirement of amino acids for growth and the faster protein turnover rate in fetal than in young or adult animals increase the relative demand for both essential amino acids and total nitrogen. Thus although an adult requires only 19% of the nitrogen supply as essential amino acids, this is increased to 43% in newborn infants.

b. Enzyme Maturation

Early in its life the fetus is dependent for its needs on a nutritionally balanced supply from the mother of amino acids and also carbohydrates and lipids. It is not until maturation of specific enzyme systems that the fetus possesses the potential to modify the supply to comply with its own requirements. Study of enzyme maturation is therefore important and classification of amino acids into essential and non-essential groups not only differs from that of the adult but also varies with gestation.

Functional maturity is not only dependent on the ability of the relevant fetal cells to synthesize the enzyme, but also on the presence of co-factors and other regulatory substances, the presence of which may in turn be dependent on a mature enzyme system. This is illustrated in the maturation of phenylalanine hydroxylase

which has been extensively investigated because of its definitive role in PKU.[37,126-133] In human fetal liver phenylalanine hydroxylase has been reported to be present at 12 weeks gestation, but lack of reduced cofactor, tetrahydropterin, prevents its expression *in vivo*. However, in similar experiments using fetal brain tissue no tyrosine formation was found, implying lack of enzyme.[126] Although lack of cofactor is the primary factor preventing expression of phenylalanine hydroxylase *in vivo*, its lack is probably related to immaturity of dihydropterin reductase, which has been shown to be lower in fetal than maternal liver.[130]

Another enzyme in the phenylalanine metabolic pathway which is functionally late maturing is tyrosine aminotransferase. Like many enzymes its activity may be regulated by the relative activities of adenylcyclase and phosphodiesterase, and consequently the concentration of cyclic AMP.[134] Studies in rats have shown that there is a considerable change in the ratio of these two secondary enzymes between days 10 and 30 postnatally, adenyl cyclase activity being relatively low and phosphodiesterase activity high before 19 days gestation. Lack of induction of this enzyme by glucagon, to which it is sensitive, may therefore be related to the low cyclic AMP concentrations, as addition of a cyclic AMP cofactor would activate the enzyme. Although 3'5' cyclic AMP may be the primary signal regulating enzyme activity it has been suggested the insulin:glucagon ratio is also important.

This ratio also changes rapidly at birth, the time at which these enzymes normally become active. Premature delivery will induce enzyme development, enzyme activities in the preterm infant being greater than those of the fetus of the same post conceptual age. However, the late developing phenylalanine hydroxylase and tyrosine aminotransferase activities can produce transient phenylalaninaemia and tyrosinaemia in preterm infants. Similarly late development of cystathionase,[135] the last enzyme of the transsulphuration pathway of methionine to cystine can lead to high plasma methionine concentrations in infants on high protein diets.

Arginine and histidine are also inadequately synthesized by the fetus and newborn, and like tyrosine and cystine are essential amino acids during this period.[136] It has been suggested that the presence of the urea cycle in the fetus is primarily to increase arginine production. Also activity of arginine synthetase in rat brain is higher in the last trimester of pregnancy than after birth. Actual enzyme concentrations are controlled not only by synthesis but also degradation rates,[137] but initial appearance of activity depends on initiating factors. As the initiating factor e.g. cortisol, glucagon, thyroxine for enzymic activity may be common for many enzymes, it is not surprising that clusters of enzymes become functional simultaneously.[138] Thus in rats hepatic aminotransferases and deaminases have been found to develop sequentially in clusters, e.g. aspartic amino transferase and arginase develop three days prior to birth, serine dehydratase and aspariginase at birth and alanine and ornithine aminotransferases in the third week of post natal life.

Similarly there is a "cluster" maturation of enzymes of carbohydrate metabolism, their development being important for the perinatal shifts in energy supply and utilization. In rats glycogen deposition is possible from the 18th day of gestation when glycogen synthetase becomes active, but it is not until birth that gluconeogenic enzymes e.g. phosphoenol pyruvate (PEP) carboxylase and glycerolkinase become active. Although hexokinase is present in fetal liver it is not until 15 - 18 days postnatal age that glucokinase and some fatty acid synthetic enzymes e.g. malic enzyme become active in preparation for the transition from a high fat to a high carbohydrate diet.

Although maturation of many of these enzymes can be induced before their normal time of appearance by exposure to the initiating factor, not all enzymes are responsive. For example, glucagon will induce PEP carboxylase and tyrosine aminotransferase activities but not arginase. Some enzymes require a combined sequential exposure to initiating factors, glucokinase only being activated by cortisol followed by glucagon, but for other enzymes such as arginase the other factors that must exist have not been determined.

The relative importance of these regulating factors can also change with age. Insulin is important in the regulation of glucokinase activity in adult liver, but has only a permissive and not a regulatory function in the enzymes development.

Thus the preterm birth, or abnormal exposure to the initiating factor in utero, would provoke early development of many of the enzymes, perhaps allowing the fetus or neonate to overcome its abnormal environmental state e.g. fetal malnutrition or postnatal diet.

c. Metabolic Disorders

Phenylketonuria provides a natural example for the study of placental amino acid transfer in man.

Oral and i.v phenylalanine 150 mg/Kg given to normal women[33] increased both phenylalanine and tyrosine in maternal and fetal plasma, with a ua:mv ratio of 1.29 - 2.26 (normal 2.36). This compared with a ratio 1.45 in a phenylketonuric patient, but the phenylalanine:tyrosine ratio in the normal fetus of the PKU mother was 8.16, compared with a normal ratio of 2.47. Clearly the placenta does not protect the fetus from maternal amino acid imbalance, but actually exaggerates the situation as fetal:maternal ratios are maintained. In addition, the high concentrations of phenylalanine may interfere with transfer of other amino acids, both across the placenta and across the blood brain barrier and other fetal cellular membranes.[89,134-142] This action is a probable cause of the fetal mental retardation observed both in man and experimental animals.[143,144] [145,146]

iv AMINO ACIDS AND PROTEIN SYNTHESIS

a. Protein Precursor Amino Acids

Conflicting evidence for the origin of amino acids utilized in protein synthesis has been reported. Similarly the rate and degree to which infused amino acids or amino acids of buffer medium mix with the precursor pool are disputed. As these factors can influence kinetic calculations based on S.A. measurements of the precursor, it is important to establish the relative roles of intracellular and extracellular amino acids in protein synthesis, both having been reported to be the precursor for protein synthesis. Evidence for the participation of intracellular amino acids in protein synthesis came from incubation of rat diaphragm with differing 14C tyrosine, Li et al finding constant 3H leucine concentrations only gave a constant moles incorporation figure when intracellular specific activities were employed.[144] However, other studies found that actual results deviated from the theoretical calculated rate of synthesis if a total intracellular amino acid pool was assumed, suggesting heterogeneity within the synthesis pool. Additionally, extracellular and intracellular amino acids equilibrated exponentially whereas incorporation into protein proceeded linearly.[148,149]

In contrast Hider et al (1969)[150] studying L leucine and glycine incorporation into protein of the rat extensor digitorum longus muscle found 14C incorporation ceased immediately when changing from a 14C to 3H environment.

Therefore, there must be a rapid mixing of the amino acids in the medium with those of the precursor pool - suggesting these are either of extracellular origin or that a very small intracellular pool of amino acids exists which is more rapidly labelled and depleted than the intracellular pool as a whole.

Further studies of amino acid flux into cells showed that[157] although there is a rapid flux of leucine across the membrane and calculations of synthesis rate based on precursor S.A. would be valid whether these were of extracellular of intracellular origin, glycine flux is slow and therefore would produce different values. As the inherent synthesis rate must be the same for both amino acids - from calculation it appeared that extracellular amino acids formed the precursor pool.

A model for protein synthesis has been proposed[150,152,153] in which ribonuclear protein in the cytoplasmic membrane was the site of synthesis, capable of accepting both intra and extracellular amino acids.[152]

The complex may be associated both with transport and synthesis, the bound amino acids being either selected for synthesis, or being released into the intracellular pool on dissociation of the complex, the latter offering an alternative explanation to the increased intracellular amino acid concentration observed when protein synthesis is blocked by puromycin.[154,155]

The fact that intracellular amino acids were only involved in protein synthesis when extracellular amino acids were absent or at very low concentration also suggests that although the complex may be formed from amino acids on either side of the membrane the extracellular amino acids are preferentially selected. Hormonal

regulation of protein synthesis may also be explained by the hormonal action on the amino acid membrane complex - either increasing the proportion entering the synthetic pathway and stimulating complex formation or promoting the dissociation and increasing intracellular concentrations.

The discrepancy between Li's conclusions[146] - intracellular amino acids as precursors and Hiders[150,151] - extracellular amino acids as precursors for protein synthesis may be due to differing techniques and preparation, diaphragm muscle having a greater surface area exposed to the medium and therefore allowing freer access than the more slowly equilibrating extensor digitorum longus muscle.

Recently further evidence of compartmentation of amino acids has been obtained from studies valyl t-RNA SAs, which were found to be intermediate between extracellular and intracellular SAs[156]. It has also been suggested that amino acids released during proteolysis have a competitive advantage over both free intra and extracellular amino acids for utilization in protein synthesis[157,158].

b. Turnover and Synthesis

Infusion techniques have been employed to assess turnover and synthesis rates. Synthesis rates may be calculated from the specific activities (SA) of the precursor amino acid and the SA of the incorporated amino acid (Appendix II) and catabolism from the decay of prelabelled proteins. Assuming that intracellular amino acids are the precursors for protein synthesis calculations using plasma SA would reduce the apparent synthesis rate as intracellular amino acids are the precursors for protein synthesis.

Early experiments using selenomethionine or ^{14}C carbonate (labels arginine) were unsatisfactory because recycling giving improbably long half lives.[159,160] Differences in half lives were therefore found between those calculated from specific activities and total activities, and between those based on protein synthesis and those on catabolism.[159,161]

Protein, and therefore amino acid, turnover rates alter with different dietary conditions, but not all proteins are equally affected: Low protein diets increased the half life of albumin in rat, whereas high protein diets reduced the half life, but globulin half lifes were unaffected.[162]

Amino balance is also important in determining protein synthetic rates, the addition of a single amino acid being capable of significantly increasing synthesis, particularly if amino acid supply is limited.[163]

Changes in half lives occur because of a shift in the balance between anabolism and catabolism. Millward found that a protein free diet slightly decreased the synthetic rate but increased the catabolic rate of liver proteins by 20%.[161] Starvation reduced liver protein synthesis by 20% and myofibrillar by 50% and although only a small change in liver catabolism occurred, myofibrillar catabolic rate increased by 75%.[161] Muscle protein metabolism therefore appears to be very sensitive to dietary control. Children re-utilized protein extensively whether protein was limited or not, but young rats' muscle protein gave results similar to previous lysine turnover experiments.[164] In these rats total lysine flux, equivalent to protein turnover, was 25-30g Kg^{-1} day^{-1}, decreasing with increased body weight and age in the male but not in the female. Six week protein depletion, but not alloxan diabetes, reduced the flux by 30%. Fetal

protein turnover rates are higher than those of the neonate, which are higher than adult values,[86] and Meier[165] finding that in the fetus large proportion of synthesis is devoted to turnover.

c. Serum Albumin

It is synthesised in the liver at one third of its capacity but extracellular albumin is secreted immediately and only occurs momentarily, if at all, in the cell. Detectable in man at 32 days gestation, with considerable synthesis between 14 and 36 weeks, the concentration increases just prior to birth, then falls within a few days of birth before increasing again at two weeks. This corresponds with changing liver amino acid concentrations.[167,168,169]

Of the amino acids only tryptophan has been found to be albumin bound, normally one tenth of the albumin molecules having tryptophan attached.[170] The only report of free tryptophan is in the newborn rat and this was ascribed to discrete changes at the association site on the albumin molecules for tryptophan. Fatty acids compete for the tryptophan binding site,[171] which could be important in gluconeogenesis and energy control. 14 g/day in man are degraded and reutilized - i.e. a major amino acid source. Albumin synthesis is very sensitive, both *in vivo* and *in vitro*,[172,173] to protein or amino acid deficiencies. During the first week of total starvation the catabolic rate remains high although synthesis declines rapidly, but on refeeding the reverse is true, synthesis increasing before catabolism - thus ensuring a rapid net increase in albumin.[174,175] However, albumin catabolism was shown to be less in malnourished infants than in those recovered from malnutrition[176] and it was suggested regeneration of albumin may be through decreased catabolism than synthesis.

The rapidity with which albumin is labelled in the liver, (serum albumin is labelled after only 10 - 15 minutes)[177], results in albumin accounting for a considerable percentage of the labelled plasma proteins after only short periods of infusion. ^{14}C leucine tracer studies of albumin synthesis show newly formed albumin to be present in the rough surface microsomes, passing after 3 - 4 minutes into smooth endoplasmic reticulum before release approximately 15 minutes later.[178] Glaumann, 1970[179] reported similar results, with release being independent of synthesis.

However, Geller et al[180] and Judah and Nicholls[181], unlike Peters[178] and Glaumann[179] found no fraction of intracellular albumin was preferentially labelled, this being in agreement with Schreiber et al[182] and Rotermund[183] who found plasma albumin increased synchronously with a decrease in smooth endoplasmic reticulum activity.

Judah and Nicholls[181] found that the synthesis of albumin involved production of an albumin precursor - proalbumin - which, although immunologically inseparable from albumin, had an oligopeptide, released by tryptic digestion, on the N terminal end.[184] It was further reported that the conversion of precursor to albumin was the rate limiting step of albumin secretion, the precursor being the most rapidly turned over and major fraction accounting for 10 - 20% of total activity.[181] The amino acid sequence of the polypeptide was found to be arginine - glycine - valine - phenylalanine - arginine - arginine,[185,186] and a pentapeptide glycine - valine - phenylalanine - arginine - arginine has been reported at the N terminal end of albumin in rat liver microsomes.[187] Further evidence of an albumin precursor has been reported in human[188] and rat liver cell suspensions.[189]

v ENDOCRINE CONTROL OF GROWTH

A brief synopsis of endocrine mechanisms for growth control in the fetus is given in order that the position of insulin may be related to overall hormonal control.

Growth hormone[4,190] does not cross the placenta and appears to have little influence on fetal growth.

Human chorionic gonadotrophin and human placental lactogen (HPL) present at very low concentrations in the fetus appear to have little influence on growth, although placental weight correlates with HPL release.[191]

Oestrogens correlate with placental weight. Oestriol concentration - and the adherence to a normal pattern of increasing concentration with gestation - particularly from 26 weeks to term, have been shown to correlate with fetal birth weight.

Androgens

Testosterone, secreted from the 9th to the 15th week of pregnancy is essential for the development of the Wolffian duct and normal male genitalia.[192,193] The slightly greater mean birth weight of males compared with females of the same gestation would suggest that fetal androgens have a similar effect on fetal growth as they have at puberty in the adolescent male.

Corticosteroids[191]

An accelerated increase at 35-37 weeks gestation correlates with storage of glycogen in the liver and maturation of the lung, suggesting that the production of glycogen and lung surfactant might be under corticosteroid control.

Renal Hormonal Function

The birth weights of anephric infants are low,[194] possibly being caused by 1:25 hydroxycholecaliferol deficiency and reduced fetal swallowing of amniotic fluid.

Pituitary Hormonal Function

Species variation is apparent in the fetal requirement for a normal functioning pituitary gland. In man evidence (derived from anencephalics and acephalics) for a hypothalmic - anterior pituitary axis control of growth is equivocal but in lambs the anterior pituitary is essential for normal fetal growth and functions independently of the hypothalmus, its absence resulting in hypothyroid lambs.[4,190] If the pituitary is absent the adrenal is most reduced in size and the thyroid least. Absence of the adrenals has no effect on lambs birth weight but may have a slight affect on birth weight in the human.

Thyroxine

Absence of the thyroid has an effect on growth in some species of long gestation.[5,194] Athyrotic fetal rats do not show any signs of thyroxine deficiency possibly because of thyroxine's relatively long half life and the rats short gestation. Lambs with the thyroid removed at 80-96 days have reduced body and skeletal growth with delayed maturation and skin development. In man the evidence is less clear. Thyroxine is released in response to thyrotrophic hormones released from the pituitary. Chorionic thyrotrophic hormone, produced in small amounts in man but not sheep, may be responsible for some of the species difference observed in fetal development related to pituitary absence. Also placental transfer of thyroxine may occur in some individuals in some species, there being no transfer in lambs but a limited transfer in the rhesus monkey.

Insulin

Insulin is known to have an important role in fetal growth and both maternal hyperglycaemia and hypoglycaemia are associated with an increased rate of fetal and perinatal mortality.[195] Maternal pancreatic insulin increased during pregnancy but maternal insulin does not reach the fetus.[196] Infants of diabetic mothers are macrosomatic withstanding acute hypoglycaemia well, but if poor maternal diabetic control results in chronic hypoglycaemia a decreased growth rate could occur.[34] Infants with pancreatic agenesis who therefore had little or no insulin during fetal development, were growth retarded, with a reduced content of DNA in muscle, liver and brain, a reduced protein:DNA ratio in muscle and an enlarged fatty liver.[197]

Insulin production and secretion is independent of hypothalmic and pituitary control, although growth hormone and an intact hypothalmus may have a permissive role in fetal pancreatic development.[4,190] A normal human fetus of a normal mother has 2% endocrine tissue, of which 40% are β cells in the pancreas. Cord blood has an insulin concentration of 8.2 ± 2.8 uU/ml.[198] In infants of gestational diabetic mothers endocrine tissue is increased to 8 - 10% of which 60% is β cell tissue and cord insulin values are $53.5 \pm 29.4\mu$U/ml. The percentage endocrine tissue and increases in cord insulin are directly proportional to the excess in birth weight. However, whereas anencephalic infants of normal mothers have normal pancreatic development, anencephalic infants (with no residual hypophysis or hypothalmus) of gestational diabetic mothers do not show the expected increase in pancreatic endocrine tissue and insulin secretion. Thus modification of the β cell response to glucose by the hypothalmus of hypophysis could be a controlling factor.

Insulin secretion in adults is regulated by nutritional, hormonal and neurohumoral action.[199,200] The actual release mechanism Malaisse suggests has three components, recognition of glucose (or other stimulus) by the cell, increased intracellular K^+ and Ca^{2+} and finally release of insulin. Cyclic AMP may have a secondary role and ATP, ADP ratio or concentrations may provide a feedback control. Fetal release is also dependent on maturity of the release mechanism. Response to the insulin secreted may also vary - end organ sensitivity, insulin degrading activity and corticotrophin - glucagon release all influencing the degree of response. The insulin release response differs from species to species[201,202] and may vary with gestational age. In man although insulin is present in fetal pancreatic islets by 11 weeks there is normally no response to glucose until 32 weeks gestation.[195] In fetal lambs insulin is present in islets and can be detected in plasma at 42 days. Differences in gestation may explain the lack of response which have been reported in man[203,204,205] and lambs.[206] Although the insulin release has been found to correlate with glucose in both species[207,208,209,210] the response is slower i.e. less sensitive than in the adult. The slow increase of plasma insulin during infusion of glucose and to a lesser degree with fructose in fetal lambs[210] (unlike the newborn in which there is a rapid rise of insulin) was unaltered by glucagon, isoprenaline and aminophylline suggesting that the cyclic 3'5' AMP dependent part of the insulin secretory mechanism may not be developed until near term. Correlation of insulin and glucose during surgery on the fetus also suggests that either the adrenal medulla does not respond to surgical stress with adrenalin release or that insulin secretion is insensitive to adrenalin inhibition.

Although arginine produces a brisk transient increase of glucagon in fetal plasma (though no glucose increase accompanies it) neither hyper nor hypoglycaemia results in any change in an exteriorized lamb fetus.[211] These findings suggest that there might be differences in maturation of responses to various stimuli for glucagon secretion. At insulin levels 20-50 times the normal physiological range glucose utilization increased and concentration decreased in fetal lambs, but umbilical blood flow, fetal oxygen consumption, maternal glucose and placental urea clearance were unchanged.[212] Smaller doses had little effect.

The duration rather than the degree of hyperglycaemia may be important in the human fetus, prolonged hyperglycaemia resulting in fetal insulin release.[213]

In the human infant insulin secretion in response to hyperglycaemia is delayed, though tissue response to insulin is probably normal,[214] and at term plasma levels (5 μU/ml) exceed normal adult fasting levels. At delivery, fetal glucose is 70-80% that of maternal values, but then decreases rapidly during the first two hours after birth. Glycogenolysis in liver is depressed to 80-90% for two hours but in muscle the decrease is greater, 50-85% and lasts longer, 1 - 3 days. Exogenous glucagon will increase blood sugar and decrease free fatty acids, therefore although the mechanism is intact, gluconeogenesis is inadequate. Gluconeogenesis from amino acids increases only slowly and increased catecholamine secretion results in the immediate post-natal secondary energy substrate being fat-plasma free fatty acids, glycerol and ketones peaking at 24-48 hours. The high amino acid concentrations during parenteral infusion of L amino acids may resemble the hyperaminoacidaemia of obesity. In adults this hyperaminoacidaemia correlated with insulin,[215] However, despite a greater increment in

insulin concentration on infusing glucose only the same decrease of amino acids was noted. This exaggeration of the β cell response could be due to either continual stimulation (because of hyperamino-acidaemia) causing secondary changes, or to the combined exogenous and enhanced endogenous amino acids resulting in increased secretion.[216]

Transfused infants with increased blood glucose and preterm infants infused with arginine (0.5 g/kg) had an increased plasma[217] insulin concentration, the latter also having increased plasma growth hormone concentrations. Infants parenterally fed with high concentration glucose solutions have a transient increase in blood insulin levels - which rapidly return to normal.[218] However, sudden withdrawal of glucose rapidly produces hypoglycaemia indicating that adaptation to the high glucose intake had occurred. In preterm infants the initial response is slow, but becomes brisker within a few days.[219]

The site of insulin action in target tissues has been found to be associated with the cell membrane.[220] The binding site is saturable, the reversible binding reaction being time and temperature dependent.[221-226] In the placenta both a high affinity low capacity and low affinity high capacity binding site have been reported,[226] the sites being relatively exposed. Small differences between species in binding site characteristics observed in placental but not liver or adipose tissue probably reflect differences in adjacent membrane structures, but differences in binding capacity also occur - rabbit, rat, mouse and hamster having only low capacity and guinea pig and monkey having a higher capacity similar to the human situation. Insulin degrading activity - two soluble enzymes and an acid precipitated enzyme - were found in placental tissue.[227]

Precipitated enzyme, probably membrane bound and closely linked to the receptor site, might solubulise the insulin (and probably glucagon) complex, rendering it sensitive to the two soluble enzymes. Insulin degrading activity in the placenta may account for the diminished sensitivity to exogenous insulin in pregnancy, particularly in the latter half, and may be implicated in the regulation of overall metabolic economy. Its action may also contribute to gestational diabetes. In addition it may prevent, again particularly in the later stages of pregnancy, intact insulin reaching the fetus, the physical barrier role present in early pregnancy being replaced by enzymatic activity.

vi AMINO ACIDS AND INSULIN

Amino acids may be utilised in protein synthesis, participate in transamination reactions or be oxidised to carbon dioxide. Availability of energy substrates, and individual amino acids, as well as the hormonal status of the intact animal are important in not only determining the relative entry rates of amino acids into these pathways, but also in controlling the proteolytic rate to meet basic metabolic demands.

a. Amino Acid Metabolism: its relationship with energy balance

Catabolism

Amino acids are important gluconeogenic precursors and in circumstances where energy substrate supply is limited will be diverted from synthetic to catabolic pathways. Study of control mechanisms has been largely confined to adult animal studies, but the poor musculature and wasted appearance of light for date infants, in which, possibly, placenta substrate transfer may have been inadequate for growth, suggest that similar mechanisms might operate in the fetus in utero. Postnatally, the relatively high caloric requirement and immaturity of the preterm infant may result in a prolonged catabolic phase.

Glucose production and release from the liver is maintained at the expense of muscle mass. In adults 50% of hepatic glucose output

is from amino acids, but in the fetus a continual as opposed to sporadic post prandial nutrient supply may alter this proportion. However, if the placental supply fails, recycling of amino acids from muscle to liver would be necessary.

Transamination reactions normally result in the predominant release of alanine and glutamine,[230] a large proportion of the latter subsequently undergoing transamination with pyruvate in the gut to form alanine.[231] During starvation hepatic uptake of alanine (along with lactate, pyruvate and nine other amino acids)[230] is increased. This lowers the arterial alanine concentration and thereby reduces the subsequent uptake, thus sparing amino acids from excessive catabolism and promoting an increase in fatty acid metabolism. Renal retention of alanine also helps the energy balance.[232] Iso-caloric protein free diets, however, result in a raised alanine concentration as gluconeogenesis reactions are limited.[233]

Transamination in the muscle and gut of amino acids and pyruvate, followed by release of the resultant alanine would not give any net gain of gluconeogenic precursors after deamination of alanine in the liver. Transamination of other TCA cycle intermediates must therefore occur.[229] In addition to its role in gluconeogenesis the alanine released from muscle may be part of an ammonia transport system between muscle and liver.[234,235,236]

Short term fasting in obese and normal adults produces a transient increase in plasma branched-chain amino acid concentrations alpha aminobutyric acid, phenylalanine, tyrosine and blood ketones, these all being very sensitive to insulin changes.[232] Rapid onset of hypoinsulinaemia in the first three days of starvation may be the basic cause of this initial hyperaminoacidaemia.[237] Plasma branched-chain amino acid concentrations may also increase because of increased competition between branched-chain amino acids and the increased concentration of non esterified fatty acids for coenzyme A. With

continued fasting, amino acids in plasma fall with the exception of threonine, serine and glycine which increase, possibly because of diminished utilisation of glycine in nucleic acid synthesis.[238,239]

Pregnancy exaggerates and accelerates these responses, an immediate increase in glycine and later threonine and serine, and a rapid fall in other amino acids - particularly alanine being apparent.

Exercise will also increase both alanine and pyruvate concentrations[240] and may release insulin previously bound in muscle tissue.[241] BCAA are selectively taken up by muscle and brain but not liver.[231]

During periods of limited substrate supply oxidation of BCAA also occurs in muscle, but not liver or brain.[242,243] Changes in oxidation and protein synthesis rates are independent of each other and are not synchronised.[244] Oxidation increased 24 hours prior to a decrease in protein synthesis in fasted rats, although both returned to control values with 24 hours of refeeding.[242] Fatty acids will stimulate oxidation but this effect can be inhibited by albumin.[245]

Inhibition of oxidation by ketones and alanine may be particularly important for nitrogen preservation during prolonged starvation and in diabetes rapid BCAA oxidation may limit gluconeogenic precursor supplies.[246]

When nutrition is adequate and amino acids are not required for gluconeogenesis, they may be directed into synthetic pathways. This situation is essential in the fetus and neonate if normal growth and maturation are to occur.

Anabolism

Increased net protein synthesis can result from increased synthesis, decreased catabolism or a combination of both.

The concentration of amino acids themselves has been shown to exert a powerful effect on the rate of protein synthesis. Increased amino acid incorporation into protein has been detected in *in vitro* preparation of rat liver, heart, muscle, and of human skeletal muscle when these preparations were incubated or perfused with amino acid enriched media.[247,248] [249,250] [246,251,252] [253,254] Jefferson and Korner,[247] reported that increased incorporation into rat liver protein was dependent upon the availability of eleven amino acids, but Buse and Reid[252] found leucine availability alone to be responsible for the stimulation of lysine incorporation into rat diaphragm protein. Differences in technique and possibly in Jefferson's study, some amino acids being present in concentrations near to their limiting concentration, could explain some of the discrepancies. The stimulation by isoleucine plus valine in addition to leucine may be because of increased competition for the enzyme alpha-ketodehydrogenase catalyzing the degradative step common to all three amino acids.[246] A leucine sparing mechanism might also account for the enhanced incorporation observed[252] in previous experiments with palmitate and acetate.[245,251] The observation that the intracellular leucine concentration of rat hearts decreased to less than 0.21 mM without glucose or palmitate in the perfusing medium, and that under these conditions incorporation of amino acids into protein was low is in agreement with a regulatory role of leucine.[255] This role, however, is disputed by Millward *et al* (1976)[256] who argues that free BCAA are raised in skeletal muscle in starvation and diabetes

but decreased in a perfused rat hemi-corpus in which protein synthesis was stimulated by insulin. BCAA would in fact be expected to increase under conditions promoting proteolysis as there is a relative abundance of BCAA in protein compared with concentrations in the free amino acid pool. An increase in oxidation of BCAA - which occurred 24 hours prior to a decrease in rat protein synthesis in Goldberg's experiment[242] could decrease the amino acid concentrations. Thus depletion of a critical compartment rather than the total free amino acid pool may be the signal for proteolysis to accelerate. Protein synthesis may be accelerated through a decrease in the catabolic rate.

Increased amino acid concentrations will inhibit normal amino acid release from tissues both *in vitro*[257] and *in vivo*[252,253], this again being associated with leucine and BCAA concentrations[246,252]. An increase *in vivo* of a rate limiting amino acid for protein synthesis, or an increase in arterial alanine concentrations because of inhibited or reduced hepatic conversion of alanine to glucose may provide feedback loops to limit proleolysis.[258]

Alternatively, or in addition high amino acid concentrations may protect the cell against increased lysosomal activity[259], a significant positive correlation between cathepsin D concentration and protein degradation[253] supporting this concept of stabilisation of the lysosomes.

The stimulatory action of amino acids may operate through their influence on ribosomes.[260] Rat liver polysomes had only a limited capacity to incorporate ^{14}C leucine into protein if no amino acids were present in the incubation medium[261], but a greater capacity was demonstrated if a complete mix of amino acids were included. Possibly the availability of certain amino acids, which may vary

depending on the type of protein to be synthesised could stimulate ribosomes to become or remain attached to mRNA - polysome profiles of liver incubated without amino acids showing a decrease in large aggregates and an increase in monomers and dimers compared to controls with amino acids present,[247] and liver ribosomes of fasted rabbits being disaggregated.[262] Stimulation appears to be due to a factor in "cell sap" rather than the ribosomes themselves,[263] with the action being subsequent to amino acyl tRNA formation.[264,265] It is suggested that stimulation of inactive ribosomes with tRNA already attached may be responsible for increased synthesis, unit synthesis per ribosome being increased.

In addition to increased availability of amino acids for protein synthesis, in the well nourished animal there is a more effective stimulus for release of anabolic hormones.

b. Amino Acid Metabolism: protein synthesis and its relationship with insulin

Insulin has been shown to stimulate the cellular uptake of amino acids[266,267,268,269] and to increase protein synthesis in vitro[154,270,271,272,273], intracellular amino acid concentrations increasing if protein synthesis was inhibited or crocodile tissue with a slow metabolic rate was employed.[274]

Uptake of amino acids may be promoted through an increased affinity for or synthesis of a carrier protein as a time dependent inhibition of the stimulatory action of insulin in vitro has been observed.[272,275,276,277] In chick embryo hearts AIB uptake was shown to be by[268,269] both a saturable and non saturable system, but only the former was

stimulated by insulin.[278] In rat calvaria Phang demonstrated cyclic AMP would stimulate uptake and suggested that the same mechanism may be involved although cyclic AMP acted at a different site to that of insulin. Increased utilization of amino acids in protein synthesis or accelerated oxidation[268] may increase uptake by reducing the concentration gradient between intra and extracellular fluids.

Although increased intracellular amino acid concentrations could themselves stimulate protein synthesis, it has been shown that this is not the sole mechanism by which insulin promotes protein synthesis. However, high amino acid concentrations have been found to impair the *in vitro* response of rat[279] and human[253] skeletal muscle to insulin, in the latter study amino acids at six times normal plasma concentrations diminishing the enhanced incorporation of amino acids from 133% to 116% the rate without insulin, whilst high amino acid concentrations increased the rate by 75%. An investigation into the relationships between *in vitro* and *in vivo* insulin stimulation was made by Lundholm and Schersten,[254] who infused glucose or saline into patients prior to muscle biopsy. Subsequent incubation at normal and high amino acid concentrations showed leucine incorporation to be the same in both tissues, with no significant difference in the percentage stimulation by insulin at the two concentrations of amino acids. This would indicate that factors other than amino acids supply must be relevant as the stimulation of amino acid *in vitro* should have been greater in the saline infused tissue if this were the sole mechanism.

This is supported by the observation that incorporation of amino acids into tissue was stimulated by insulin during incubation in a Krebs-Ringer buffer[246] without glucose or amino acid supplementation.

Additionally the greater correlation found between $L^{14}C$ leucine incorporation into human skeletal muscle and RNA concentration in basal and insulin stimulated conditions than when stimulation was with high amino acid concentrations indicates insulin has a secondary separate action on protein synthesis.[151]

Insulin will influence the rate of proteolysis but it is not as potent a stimulus as amino acids, which will reduce proteolysis when there is already maximum inhibition with insulin.[257] The effect is also altered by prevailing substrate supplies.[246] Fulks found that insulin produced equal effects on degradation and synthesis in normal and fasted rat diaphragm, but glucose only enhanced the insulin inhibition of proteolysis. However, although cyclohexidine increased protein turnover rate by 50%, Rannels [258] et al demonstrated that the percentage inhibition if insulin was present was the same. In vivo, infusion of insulin produces a marked decline in plasma BCAA methionine tyrosine and phenylalanine, suggesting a reduced release by muscle.[231]

Stimulation of lipogenesis by insulin, like proteolysis is dependent on the energy balance.[243] In fasted and control rat tissues glucose and insulin were effective, but there was no response in fasted refed rats in which both lipogenesis and leucine concentrations were already increased.

In pancreactectomised rats, insulin stimulated leucine incorporation into liver proteins.[280] Similarly, diabetic muscle has been found to have a reduced capacity for incorporating methionine into protein[281] and to have fewer normal polysomes than control tissue[282]

both of these defects being corrected by insulin. Addition of glucose to diabetic rat tissue in vitro increased incorporation of glycine into glutathione towards normal and with the addition of insulin, normal control rates were achieved.[283] However, without substrate and in normal tissue insulin had no effect and in tissue from starved but normal rats glucose but not insulin was necessary to achieve normal glycine incorporation. Both adequate substrate and insulin (which possibly has a delayed action) appeared necessary for protein synthesis. Similarly increased glucose utilization, glycogen formation and ^{14}C alanine incorporation into protein in insulin treated rat diaphragm was supressed by glucose addition.[284] Insulin stimulation of protein synthesis however, is not dependent on glucose concentrations, stimulation after depletion of endogenous substrate being the same as in control tissue.[285,286]

The infusion of glucose or insulin creates in part the status of the fed animal. In man, infusion of glucose (25 mg/Kg/hr) causing a five fold increase in plasma insulin almost completely inhibited hepatic glucose output and reduced the fractional extraction rate of amino acids.[230] Slower glucose infusion (2 mg/Kg/hr) reduced hepatic glucose output by 85% but other parameters remained unaltered.[287,288] Similar results were found in muscle of man and newborn lambs.[289]

In contrast, studies on hepatic glucose metabolism in adult ruminants[290] found that although insulin controlled gluconeogenesis in peripheral tissues, glucagon alone was responsible for the increased hepatic uptake of amino acids for gluconeogenesis. The apparent effect of insulin in non ruminants, it was suggested, was caused by decreased glucagon secretion during the glucose infusions employed

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to increase insulin concentrations. Prior also found that glucose infusion would decrease plasma essential amino acids to 65% and non essential amino acids to 81% of control values in non pregnant ewes but that exogenous insulin given either as an injection or continuous infusion had little effect on concentrations. Simple provision of an energy source did not appear to be the explanation as acetate was less effective than glucose.

Failure to halt hepatic output of glucose in insulin dependent
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diabetics despite high glucose levels would also indicate that glucose alone cannot be the controlling factor for gluconeogenesis. However liver appears to be the major organ maintaining homeostasis and its sensitivity may be enhanced by the relatively greater insulin and glucagon concentration in portal than peripheral blood.

Thus, although insulin is possibly the major hormone for the control of glucose homeostasis there appear to be quantitative differences in the response of the homeostatic control mechanisms. Also, the sensitivity of hepatic tissue to insulin might be less in the newborn. (See page 38).

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Changes in plasma glucose with insulin may not be apparent as the red blood cell may carry glucose from the capillary bed of muscle when insulin concentration is low, but release glucose to plasma and thus to tissues when insulin concentrations increase. Likewise, plasma amino acid measurements may not accurately reflect
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tissue availability. Elwyn et al (1968) report that glutathione synthesised in gut and liver is transported to peripheral tissue via erythrocytes and a high positive association exists between hepatic protein breakdown and a high amino acid concentration and

nitrogen transport in the erythrocyte. Plasma amino acid concentrations were lower in the hepatic than portal veins of dogs but the reverse was true of erythrocytes amino acid concentrations. Likewise the greater concentration of amino acids in the portal vein than artery was reversed in erythrocytes. A large contribution by the red blood cell to net transport may therefore be present if concentrations of amino acids are high in erythrocytes and if there is a high haematocrit. Anderson (1974) found no correlation between the post prandial decrease in plasma or erythrocyte amino acid concentrations with insulin or glucose concentrations.

Chapter II

Biochemical Methods

This chapter includes laboratory methods employed during the investigation of the effect of insulin on both placental transfer in sheep and plasma amino acid concentrations in the neonate.

Individual experimental details are to be found in the respective sections for tritium, ^{14}C and insulin and ^{14}C amino acid infusions and the neonatal section.

Biochemical Analyses

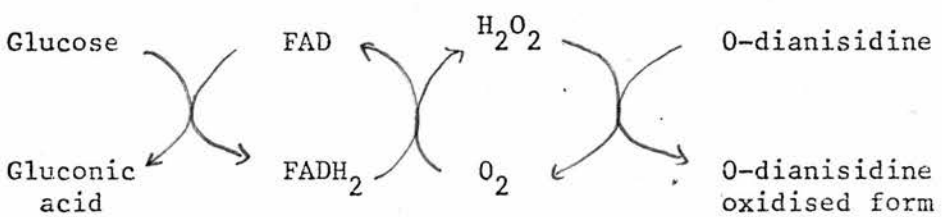
Materials Unless otherwise stated reagents were A.R. quality supplied by B.D.H.

i. Haematocrit

Microhaematocrit determinations were made using 75 mm capillary haematocrit tubes, sealed in the flame of a bunsen burner and spun in a Hawkesley microfuge for five minutes. The haematocrit value was read from the Hawkesley microhaematocrit reader.

ii. Plasma Glucose

Glucose in blood was determined by the enzymic method of Raabo and Terkildsen, 1960[295]. The method involved oxidation of glucose to gluconic acid and hydrogen peroxide by glucose oxidase (0.75 g in 250 ml phosphate buffer 6.6 mmol $NaH_2 PO_4 H_2O$ per litre) at pH 7. In the same buffer peroxidase was included (3.7 mg) so that the oxidation and hence colour production of the dye o-dianisidine (500 mg in 100 ml acetone) may be coupled to the glucose oxidation.



An enzyme dye reagent was prepared - 1 ml o-dianisidine to 150 ml enzyme solution. 400 µl polythene tubes with 100 µl saturated sodium fluoride were dried at 70°C overnight. These were more than half filled with blood and then spun in a Beckman/Spinco microfuge for 2 minutes.

20 µl plasma was pipetted into a 10 ml glass test tube and 5 ml enzyme-dye reagent added. After mixing, the tube stood at room temperature for 40 minutes out of direct sunlight before reading against a reagent blank at 500 mµ in a Pye-Unicam SP600. Glucose standard solution (5.5 mmol/l) was used to calibrate the method. Reagent, glucose and where possible sample values were determined in triplicate.

A modification of the method was used if haemolysis was present. Proteins were precipitated by the addition of 100 µl 3 per cent perchloric acid to 20 µl blood or standard and 50 µl of the supernatant was mixed with 5 ml enzyme-dye reagent and read as before.

iii. Total Proteins

Total protein was measured using a modified biuret reagent * method of O'Brien and Ibbott 1964[296] using a Beckman/Spinco Model 150 Ultramicro Analytical System.

5 µl sample, water (blank) or standard (bovine albumin 60 g/l Armour Pharmaceutical Co. Ltd.,) were pipetted in triplicate into 400 µl micro test tubes. 200 µl of the biuret reagent * and 50 µl 25 per cent sodium sulphite solution were added and the tube contents mixed well. Absorbance values at 540 mµ were read in the colorimeter after 30 minutes.

iv. Total Lipid

Plasma total lipid concentration was measured using a Boehringer Mannheim Biochemica Test Combination Kit. Plasma (0.5 ml) or 0.5 ml standard solution (1000 mg total lipid/100 ml) were mixed well with 2 ml AR concentrated sulphuric acid in a test tube. After heating in a boiling water bath

* See appendix 2

for exactly 10 minutes the tubes were cooled. 0.1 ml of these plasma or standard solutions and a further 0.1 ml AR concentrated sulphuric acid (blank) were added to 2.5 ml of the colorimetric reagent (14 mol/l phosphoric acid, 13 mmol/l vanillin) and the tubes well mixed. After standing at room temperature for 30 minutes, colour development was measured at 530 mμ on a SP600 spectrophotometer.

Sample total lipid concentration (c) was calculated from the equation:

$$C = 1000 \times \frac{E \text{ sample}}{E \text{ standard}} \quad \text{mg/100 ml} \quad (E = \text{extinction})$$

v. Non-Esterified Fatty Acids

A colormetric method using Doles extraction mixture and a cobalt reagent composition was used to measure plasma free fatty acid concentrations. (Reagent composition - Appendix 3). Plasma 50 μl was added to 250 μl Doles extraction mixture - (40:10:1 v/v/v isopropanol:hexane: 1N H_2SO_4). A blank - 40 μl water and 250 μl Doles extraction mixture - and standards 50 μl water and 250 μl Doles extraction mixture containing either 20 or 40 μmol/l palmitic acid were also prepared.

All tubes were stoppered and mixed with a whirlimixer before standing 5 minutes at -20°C. Heptane (300 μl) followed by 500 μl water were added to the tubes which were remixed before centrifuging for 2 minutes at 3000 rpm. The upper heptane phase (300 μl), 400 μl chloroform:hexane (5:1 v/v and 500 μl of the cobalt reagent were then thoroughly mixed

with a whirlimix for 3 minutes, before centrifuging the stoppered tubes at 2500 rpm for 15 minutes. 500 µl from the upper chloroform-hexane phase was then mixed with 750 µl of indicator solution (freshly prepared 0.016 per cent alpha nitroso-β-naphthol in 96 per cent AR ethanol) and the colour read 30 minutes later at 500 mµ. Non esterified fatty (NEFA) acid concentration was calculated from the formula.

$$\text{NEFA (mmol/l)} = \frac{\text{Sample reading - blank reading}}{(\text{20 mmol/l std - blank reading}) + (\text{40 mmol/l std - blank reading})} \times 0.6X5$$

vi. Osmolality

Plasma and urine osmolalities were measured on 0.2 ml samples by freezing point depression using an Advanced osmometer.

vii. Ultrafiltration

Sartorius ultrafilters and ultrafiltration apparatus were employed. 0.5 - 1 ml plasma was placed in a stainless steel pressure filter holder with a cellulose nitrate filter - Sartorius No. 121 - 36 in position. Using oxygen-free nitrogen, compounds with a molecular weight below 10,000 were forced through the filter overnight at a pressure of 40 lb/sq/in.

viii. Sephadex

0.5 or 1 ml of (plasma) was pumped on to a 50 cm G200 superfine Sephadex column at a pump rate of 7 ml/hr. Protein fractions were eluted with a phosphate buffer (Appendix 4) at pH 7.4 and collected at 30 minute intervals. The absorbance at 280 mµ was recorded.

ix. Free Amino Acid Concentration Analysis

Free amino acid concentrations were measured by ion exchange chromatography (Cockburn et al 1971)[297] using a modified Technicon Amino Acid Autoanalyser. Water jacketed twin capillary columns 140 cm in length, 6 mm internal diameter were packed with Chromasorb B resin - an anionic sulphonic acid resin of beads 17 μ ± 2 in diameter. Buffer (for composition see Appendix 5) increasing in pH from 2.85 to 6.25 was pumped at high pressure simultaneously down both columns from a nine-chambered autograd -

AUTOGRAD

Chamber 1 - 42 ml buffer pH 2.85 plus 3 ml AR methanol

Chamber 2 - 43.5 ml buffer pH 2.85 plus 1.5 ml AR methanol

Chamber 3 - 45 ml buffer pH 2.85

Chambers 4-5 - 45 ml buffer pH 3.75

Chambers 6-9 - 45 ml buffer pH 6.25

For the first 5½ hours the water jacket temperature was 34°C and thereafter 70°C resulting in pumping pressures of 600 - 700 lb/sq/in and 300 - 400 lb/sq/in respectively.

The column effluent was passed through a manifold proportionating pump before mixing with ninhydrin (composition Appendix 6) and oxygen free nitrogen. After passing through 3 m glass coils in an oil bath at 90°C the effluent was cooled in water jacketed coils before passing through 2 continuous flow colorimeters. The OD at 570 mμ and 440 mμ was charted using an Elliot 4 point multiphasing recorder. The concentration of individual free amino acids was calculated from the area under the 570 mμ peak (proline

and hydroxyproline 440 mμ) and relating this to the area of a norleucine (NLE)internal standard peak. Norleucine was used as an internal standard in all amino acid concentration determinations, the colour index i.e. relationship between the peak areas of 0.04 μmol norleucine and a known amount (usually 0.04 μmol) of each amino acid having been previously determined.

Sample Preparation

Where sample volume permitted, 0.2 ml plasma or 0.2 ml blood plus 0.2 ml H_2O were pipetted into a pyrex test tube. Fetal plasma free amino acid concentrations, because of the small volume of samples, were normally measured on 0.1 ml sample.

To the sample was added 0.05 μmoles norleucine contained in either 0.1 or 0.2 ml HCl/0.17N, the volume depending on the internal radioactivity/norleucine standard solution used (see p 65) After mixing, a volume equivalent to the sample volume i.e. 0.1 or 0.2 ml of 0.6 mol/l sulphosalicylic acid was added to the sample and the tube contents mixed by means of a whirlimix. After allowing 10 minutes for protein precipitation, the tubes were centrifuged at 3000 rpm in an MSE bench centrifuge for 5 minutes. The supernatent was decanted and the pH adjusted to pH2 with 75 μl of 2 mol/l LiOH. H_2O. The sample was applied in a 25 per cent sucrose solution to the top of one of the columns and the chromatograph run overnight. 10 μl maternal, 20 μl fetal hydrolysis samples with NLE as internal standard were applied in sucrose to the columns without pH adjustment.

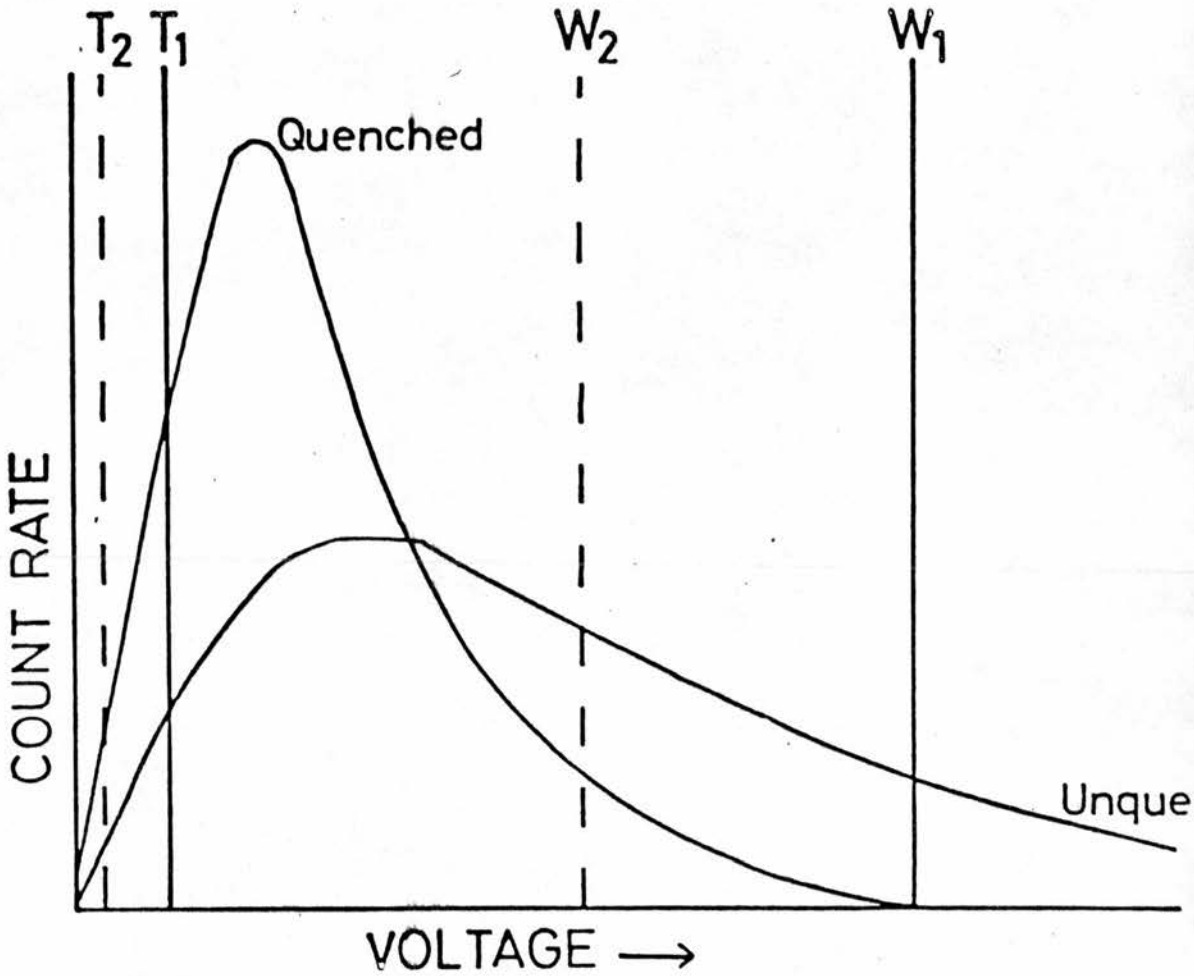


Diagram to show the effect of quenching on the pulse amplitude spectrum of an isotope

x Radioactivity Measurement

The activity (Ax) of any sample may be calculated from the formula:-

$$Ax = \frac{\text{Counts per minute recorded}}{\text{efficiency of counting.}}$$

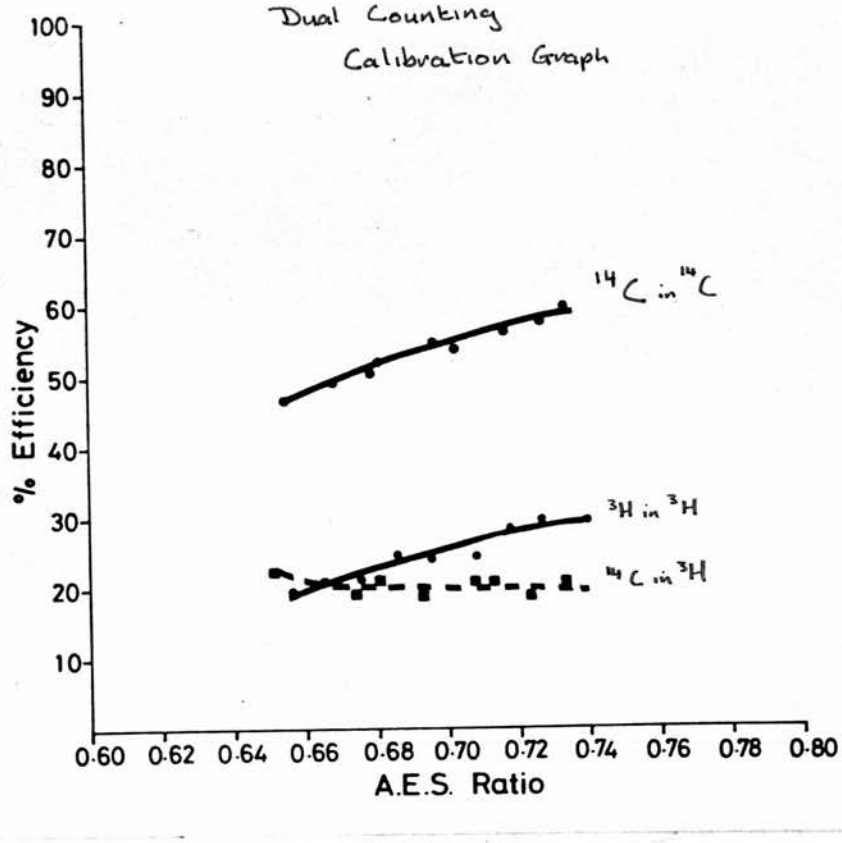
The efficiency of counting in any system is reduced if either colour or chemical quenching is present. As almost all biological materials produce quenching it is necessary to ascertain the amount of quench for each sample system. By reference to a prepared calibration graph of degree of quench versus efficiency, the activity of a sample can be determined.

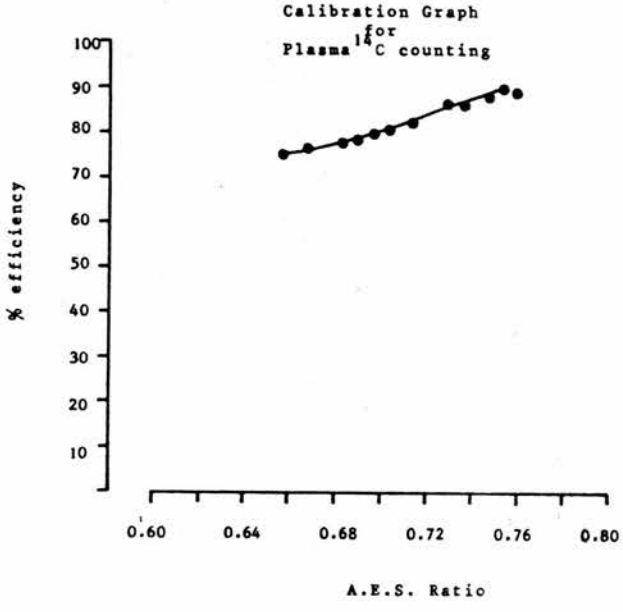
Measurement of Quench

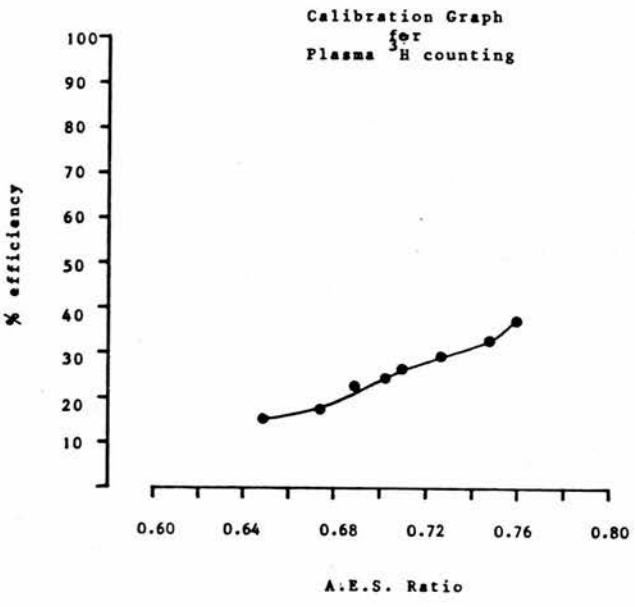
The net result of quench is to shift the amplitude spectrum of a particular isotope to lower voltage levels. Although with the ^{14}C isotope this can be partially compensated for by shifting threshold and window settings, usually by increasing the amplifier gain (in diagram 1 from T1 W1 to T2 W2) so that the spectrum is measured at a lower pulse height - the quenching of ^{3}H which is a weakly energetic isotope is not overcome.

In most experiments quenching was ascertained by use of an automatic external standard. The ratio (A.E.S. ratio) of net counts of the external standard recorded with the sample vial between the external standard and the photomultiplier detector system to the counts of the external standard alone gives a

Dual Counting
Calibration Graph







measure of quench.

Thus A.E.S. ratio =

$$\frac{\text{(C/min of external standard + c/min sample) - c/min sample}}{\text{c/min external standard.}}$$

Although two counting periods are required to ascertain the net counts of the quenched external standard, the first measurement being of c/min standard and sample and the second of c/min from the sample alone, the high activity of the external standard allows short counting periods- 1 minute. A calibration graph of the A.E.S. ratio versus efficiency ($\frac{\text{observed c/min}}{\text{expected c/min}}$) is prepared to determine the corrected activities of the samples.

Calibration Graph:

These were prepared by adding small volumes (50 μl) of either a ^{14}C or ^{3}H labelled aminoacid solution of known high activity (0.5 μ Ci/ml ^{14}C or 2 μ Ci/ml ^{3}H) to several non-radioactive samples at 0.5, 1 and 2 times the sample volume for 20 minutes in triplicate and counting these vials after addition of the relevant scintillant - NE260, NE262 or Instagel. Examples of calibration graphs for NE260 and plasma (^{3}H and ^{14}C and for dual counting, ^{3}H and ^{14}C together) are shown opposite. Calibration graphs for other scintillator - sample systems showed only minor variations.

Dual Labelled Samples

If the β spectra of two isotopes is sufficiently dissimilar it is possible to measure the relative amounts of activity from each isotope. This is done by measuring the activity at 2 window settings such that in one the isotope emitting at a lower pulse height (in these experiments ^{3}H) is measured with little contribution from the second isotope (^{14}C) which gives a pulse amplitude spectrum at a higher voltage.

Optimal settings of the window must maximise the counts in the ^{3}H window whilst minimising the counts from ^{14}C in this same window. Window settings may not therefore be at the maximum tritium count rates (i.e. efficiency) as too great a "carry-over" from ^{14}C may occur at this setting. After a trial of a variety of different window settings it was found that the factory settings gave optimal results.

Activity of ^{14}C and ^{3}H isotopes were calculated from the formula:-

$$^{14}C = \frac{\text{c/min } ^{14}\text{C channel (C1)}}{\text{Efficiency of } ^{14}\text{C in } ^{14}\text{C channel (E1)}}$$

$$^{3}H = \frac{\text{c/min } ^{3}\text{H channel } \left(\frac{\text{C1 X efficiency } ^{14}\text{C in } ^{3}\text{H}}{\text{E1}}\right)}{\text{Efficiency of } ^{3}\text{H in } ^{3}\text{H channel}}$$

The high degree of colour quenching of whole blood did not allow differentiation of ^{14}C and ^{3}H activity. The ^{14}C spectrum was shifted so that the overlap with the tritium spectrum was too great to permit separation of the spectrum

Sample Counting

A Packard 2425 scintillation counter was used for all determinations. ^{14}C samples were counted on the machine set ^{14}C windows, Gain 5 per cent lower window 20, upper window 1000 as was ^{3}H, Gain 50 per cent, Lower window 40 upper window 1000 and dual $^{14}C/^{3}H$ - machine factory pre-set. The automatic external standard was used in all cases for correction of quenching and efficiency. Samples were counted for 20 minutes.

a. PLASMA

Total Plasma Activity

Plasma 0.5 ml was pipetted into a counting vial and 10 ml NE 260 scintillator added. All maternal and fetal samples were counted.

Plasma "Water" Activity

In years I and II plasma 0.5 ml was deproteinised using 0.5 ml 0.6 M sulphosalicylic acid. The sample was whirlimixed and then stood 10 minutes at room temperature. After centrifuging in a bench centrifuge at 3000 rpm for 5 minutes, 0.5 ml of the supernatant was counted with 15 mls NE 260 as scintillator. Some fetal samples, because of the small volume of plasma available, were prepared from smaller volumes in a similar manner with the same proportions of reagents. In year III, plasma was deproteinised with solid sulphosalicylic acid - 30 mg per millilitre. After mixing, standing and centrifuging as in years I and II, the supernatant was decanted into a vial

for counting. The precipitate was washed with 1 ml distilled water and the washing process repeated. Three washings of the precipitate were carried out, and the total counts of the 4 vials added to give the plasma water content.

Plasma "Water" Specific Activity

Selected samples - 1 ml maternal plasma or 0.5 mls fetal plasma - were prepared in a manner identical to that used for amino acid determination. An internal radioactive amino acid was employed to correct the results based on recovery of that amino acid. The radioactive amino acid used varied with the amino acid infused into the sheep, the amino acid radioactive standard being chosen both to give good separation from the infused amino acid on elution and to be unrelated metabolically i.e. valine was not used for leucine infusions.

A second amino acid was used for hydrolysate corrections as shown below:

Experiment	Infused Amino Acid	"Plasma Water" int. standard	Hydrolysate int. standard
C1	Phenylalanine	Valine	Leucine
C2	Phenylalanine	Valine	-
C4	Phenylalanine	Valine	Leucine
Ins C1	Phenylalanine	Valine	
Ins C2	Leucine	Phenylalanine	
Ins C4	Lysine	Phenylalanine	
Ins C5	Lysine	Phenylalanine	

In the same radioactivity standard solution norleucine was included as the internal standard for concentration measurements, so that the same solution was used both for amino acid con-

centration and radioactivity measurements.

Composition of these standards is given below:

L-^{14}C - Valine - 0.25 µmol/ml Norleucine 12,385 d/min/ml

L-^{14}C - Leucine - 0.50 µmol/ml Norleucine 12,600 d/min/ml

L-^{14}C - Phenylalanine - 0.50 µmol/ml Norleucine 20,340 d/min/ml

The supernatant from the same precipitated sample was applied equally to both columns, concentration and radioactivity measurements on the same sample being obtained from the same overnight autograd buffer gradient. The effluent from the "specificactivity" column was diverted to a fraction collector after passing through the manifold tubing, but before mixing with ninhydrin. The time difference between the amino acid peak appearing on the recorder and the collection of the peak in the fraction collector had been previously measured, so that the amino acid recorded chart could be used to determine the contents of the fractions. Fractions were collected originally at 15 minute intervals in test tubes, and later, when an Isco model 328 fraction collector became available in Year III at 10 minute intervals directly into disposable scintillation vials.

Where necessary, fractions of approximately 3.5 ml were decanted into disposable counting vials and 15 ml NE 262 scintillator used to rinse out the test tube and add to the vial. 10 ml NE 262 scintillator was added to the fractions collected directly in the vials.

b. HYDROLYSATES

The protein precipitates from the "specific activity" measurement samples were washed by whirlimixing and centrifuging

three times with saline - 1 ml volumes for maternal and 0.5 ml volumes for fetal samples. The washings were drained into counting vials and 15 ml NE 262 added before counting to ensure radioactive material of the supernatent had been removed. The thick walled pyrex test tube which contained the precipitate was drawn/using a glass blowers flame to give a narrow neck /out through which 0.5 ml water and 0.5 ml concentrated Aristar hydrochloric acid containing 0.2 per cent phenol were added. The tube contents were frozen in an acetone/solid CO_2 bath and the tube evacuated using a vacuum pump. The contents were allowed to thaw then refrozen and the tube re-evacuated. The neck of the tube was sealed with a gentle flame while the contents were still frozen and the tube under vacuum.

Samples from one experiment were all hydrolysed together in a domestic pressure cooker at 105°C for 20 hours. Each vial was opened and the contents made up to 2 ml with distilled water in a 10 ml measuring cylinder. The contents were well mixed decanted into a small test tube and centrifuged for 30 minutes at 3000 rpm in a MSE bench centrifuge. The supernatant was stored at -70°C until analysed.

Hydrolysate Total Radioactivity

Hydrolysate 0.5 ml with 10 ml NE 260, was counted in disposable vials.

Hydrolysate Specific Activity

0.1 ml of the maternal plasma hydrolysate, or 0.4 fetal plasma hydrolysates were added to a radioactivity/norleucine standard as previously described (see p 64). The pH was adjusted with

2 mol/1 LiOH until alkaline afterwhich 60 µl [illegible] sulphosalicylic acid was added. This was put on the column and the specific activity determined as for plasma water (p [illegible])

e. WHOLE BLOOD

Total Radioactivity

Decolourisation before counting was necessary because of strong colour quenching.

Maternal

0.2 ml blood was pipetted into a counting vial and the pipette washed with 1.5 ml 1:1 v/v soluene 100 (Packard): isopropanol. The contents were swirled then 0.6 ml hydrogen peroxide (30 per cent w/v) added and the sample again well mixed. After standing for one hour at room temperature, the blood was decolourised. 15 ml of 1:9 v/v 0.1 NHC1:Instagel (Packard) was added and the yellow tinged solutions counted.

Fetal

Preparation was as for maternal samples but only 0.1 ml volume fetal blood and 0.5 ml hydrogen peroxide used.

Deproteinised Blood

0.4 ml blood were pipetted into a thick walled test tube and the pipette washed with 0.4 ml saline. The blood was deproteinised as for plasma with 0.4 ml 0.6 mol/1 sulphosalicylic acid. 15 ml of NE 262 scintillator were added to the decanted supernatant and the vial counted.

xi. Tissue Counting

Tissues were stored at -70°C and were removed one at a time for sampling. Disposable vials were preweighed to 4 decimal places.

Thin slivers of tissue were shaved with a scalpel from the frozen tissue and placed on the bottom of the preweighed vial. Vials were reweighed and the tissue weights recorded - between 40 and 200 mg depending on the tissue type, after which tissues were immediately replaced in the deep freeze.

Soluene 100 was added to the vials, 100 µl to 10 mg tissue with a minimum volume of 500 µl so as to cover the bottom of the vial. The vial was swirled and/or tapped to release any adhesions between tissue and vial. After overnight digestion at room temperature, and if necessary warming the following morning, Instagel 10 ml was added and the samples counted.

xii. Autoradiography

Prepared slides of tissue were dipped for 5 minutes in xylene, twice in ethanol and once in 70 per cent v:v ethanol: distilled water. The slides were rinsed under the tap.

In the darkroom Ilford photographic emulsion K5 (grain size 0.20 µ highest sensitivity) was prepared: 1 part 1 per cent glycerol: 2 parts emulsion in a 100 ml beaker. This was warmed without stirring under the tap until all the emulsion was dissolved. A single slide holder was filled to overflowing (to remove any airbubbles) and placed in a water bath at 43 - 45°C. The slides were dipped singly into the emulsion. After removal, the back and sides were wiped and the tissue side dried using cold air from a hairdryer. When all slides were dipped and dried they were placed in a "baffle box" - which admitted air but no light - for 2 - 3 hours to thoroughly dry. They were subsequently wrapped in tin foil and stored 3 - 6 weeks at 4°C, during which time radioactivity ionized and reduced the silver halide ($AgBr_2$-AgO) to produce black grains in the emulsion. By conventional staining with eosin (part xiii) to differentiate the cells, the distribution of activity in the tissue could be examined.

xiii Haematoxylin and Eosin. Counterstaining Autoradiographs.

Method

The fixed autoradiograph sections were washed and dried before staining for 3 minutes in Mayer's acid haemalum. After washing well in tap water the sections were dipped for 5 - 10 seconds in 1 per cent acid alcohol (1 per cent v/v HCl in 70 per cent alcohol). Sections were again washed in tap water before "blueing up" in Scott's Tap water substitute (weak alkali), afterwhich they were again washed in tap water. Counterstaining was done by staining with 1 per cent Eosin for 1 - 2 minutes. After washing, sections were dehydrated in several changes of absolute alcohol before clearing in xylene and mounting in synthetic resin.

Chapter III

ANIMAL EXPERIMENTAL METHODS AND RESULTS

This is divided into 4 major parts. Details of animal care, surgery and preliminary treatment of samples prior to storage which are common to all experiments are given in the first section.

Experimental details, results and evaluation of these for the tritium and ^{14}C labelled amino acid and insulin with ^{14}C labelled amino acid infusions are given in parts 2, 3 and 4.

A summary of haematocrit values is given for each experiment. Individual results can be seen in Appendix 6

Also in the appendices are tables summarising the data from all experiments. Appendix 7 gives the rate of increase in blood and plasma, appendix 8 the radioactivity level at plateau or maximum activity reached and appendix 9 the total plasma protein (where measured).

PART I

Experimental Methods Employed in All Experiments

The validity of *in vivo* experiments depends upon the quality of the animal preparation used in the study. Sheep employed in these experiments were housed and cared for in the Fetal Physiology Department of the Animal Diseases Research Association, Moredun. Handling and feeding techniques previously shown to produce minimal stress conditions[2,3,298,299] were used to maintain the sheep in as normal a physiological and metabolic condition as possible. The monitoring of the animal preparations, and factors affecting the physiological and metabolic state of the animals are now discussed.

Physiological Monitoring

The physiological state of both the mother and catheterised fetus must be monitored to ensure that the following criteria are fulfilled before commencing metabolic studies:

a) the stress effects of anaesthesia and surgery have passed

b) the physiological state is stable and not deteriorating

and c) a metabolic steady state has been achieved.

The sheep in these experiments were maintained at the Moredun Research Institute at the same time and under the same conditions as similar preparation which were used to study various aspects of fetal physiology and to define when the above criteria have been established.[70,300-302] The sheep were at least 11 and usually 14 days passed the time of surgery* and therefore the first condition was satisfied. Regular PCV, glucose fructose, insulin and some other parameters showed little variation after any changes associated with surgery, and all values were within the well established ranges for preparations of this type.**

* see page 204

** personal communication from Dr Mellor

TABLE II:

	Experiment			
	C1	C2	C3	C4
Amino Acid Infused	Phe	Leu	Phe	Phe
Infusion Route	M	M	F	M + F
Gestation* (days)	126	130	127	126
Days post surgery	20	16	15	16
Lamb weight** (Kg)	3.52	3.47	3.49	3.47
Placental weight (g)	433	517	607	358
++PCV %				
M 27.1 ± 1.0	25.0	31.0	30.0	27.5
F 36.7 ± 0.09	34.0	32.0	28.0	35.5
+Glucose (mmol/1)				
M 2.87 ± 0.41	2.99	3.67	2.73	3.63
F 0.74 ± 0.18	0.65	0.96	0.76	1.04
+Fructose				
M				
F 4.76 ± 1.25	6.49	5.11	3.66	6.32
+Amino Acid (μmol/1)				
M phe 35 ± 9	26.9			33.8
F phe 88 ± 22	79.2		48.3	69.1
M leu 67 ± 26				
F leu 106 ± 34		92.4		

M = Maternal
F = Fetal
Phe = phenylalanine
Leu = leucine

* gestation at time of experiment
** corrected lamb weight for time of experiment (weight gain = 73 gms per day) Mellor and Murray (1981)[304]

\+ Slater and Mellor (1979)[70]
++ Mellor Matheson and Small (1979)[303]

TABLE III i

	Experiment				
	Ins C1	Ins C2	Ins C3	Ins C4	Ins C5
Amino Acid Infused	Lys	Phe	Leu	Lys	Lys
Infusion route	M	F	F	F	F
Gestation (days)	122	121	126	125	124
Days post surgery	14	11	16	15	15
Lamb Weight (Kg)	2.74	2.16 2.11	3.65	2.91	2.94
Placental weight (g)	334	242	405	376	363
+++ PCV %					
M 27.1 ± 1.0	32.5	28.5	32.0	32.0	32.5
F 36.7 ± 0.9	39.0	34.5	37.0	37.0	38.5
+ Glucose (mmol/l)					
M 2.87 ± 0.41	3.47	2.99	3.11	3.44	3.27
F 0.74 ± 0.18	0.99	0.80	1.44	0.85	1.44
++ Insulin (ng/ml)					
M 0.37 ± 0.014	1.37	0.65	1.00	1.37	0.43
F 0.34 ± 0.015	0.63	0.57	1.03	0.63	0.59
+ Amino Acid (μmol/l)					
M phe 35 ± 9					
F phe 88 ± 22		85.2			
M leu 67 ± 26					
F leu 106 ± 34			81.1		
M lys 131 ± 37	82.0				
F lys 113 ± 36				56.5	74.2

M = Maternal

F = fetal

Phe = phenylalanine

Leu = leucine

Lys = lysine

+ Slater and Mellor (1979)[70]

++ Slater and Mellor (1981)[300]

+++ Mellor, Matheson and Small (1979)[303]

Values for these parameters prior to starting experiments are given in Tables II, i and ii , together with published values established at the same centre.[70,300-303] Furthermore, studies from the centre have shown that there is little variation in the mean of these parameters either within a year or between years.

Thus as no value fell outside the range observed in successfully catheterised fetuses in a stable physiological state it is reasonable to assume that these animal preparations also met the required criteria. Metabolic steady state was achieved by introducing hourly feeding at least 7 days prior to experiment.

Although all these fetuses had values for the measured parameters well within the ranges, it should be noted that the naturally occurring wide range of placental weights,[303] which can influence parameters results in a wide range for these values. Failure to recognise the effects of the wide range of placental weights could therefore result in some preparations being judged "abnormal" when in reality their condition was "normal" for a placenta of that size. Thus the concept of normality is difficult to apply, and the suitability of animal preparations should be judged by the aforementioned criteria. Comparison of data between centres may therefore not be possible and any parameters e.g. pH pCO_2 pO_2 and lactate,[304,305] in addition to those used in these experiments, may be used, provided that the stable state can be defined.

Plasma amino acid concentrations were not examined until the start of the experiment, a time when the lowest concentrations are observed (i.e. approximate -20 days from birth). From data of previous similar experiments (with the exception of once daily feeding)[70] mean phenylalanine concentrations ($\pm$ SD) would be between 70 and 76 umol/l and leucine and lysine concentrations between 81 and 100 umol/l and 69 and 93 umol/l respectively. Values for phenylalanine leucine and lysine plasma

free amino acid concentrations in hourly fed fetuses between 125 and 134 days gestation were 93 ± 1.2 (S.E.M.) 80 ± 1.9 and 78 ± 2.0 umol/l respectively.[300]

In conclusion, as all values of the measured parameters were reasonably constant and fell within the expected range for this type of animal preparation, it was assumed that a stable physiological state had been attained. Additionally sufficient time elapsed for recovery from surgery and to establish steady state metabolism after commencing hourly feeding.

Pre-experiment care preparation and conditions

Scottish Black Face ewes were housed in an unheated room temperature 12-17°C with constant artificial light. Here they were handled daily for 4 - 8 weeks prior to surgery. They were fed Ruminant A diet (Seafield Mill, Midlothian) which was divided into 24 portions, each portion being delivered to them every hour by an automatic feeding device.

Surgery

At approximately 105 - 110 days gestation, umbilical arterial and venous catheters were inserted by Dr. Mellor[3,306].

Two weeks before surgery the sheep was completely shorn posterior to the last rib and 48 hours before surgery food, but not water was withheld. Anaesthesia was induced with IV pentobarbitone sodium and 100 per cent oxygen was administered by endotracheal tube. Surgery was performed under sterile conditions in a surgical theatre. Iodine antiseptic solution 0.05 per cent w/v chlorhexidine and 0.1 per cent w/v thiomersal solution (50:50 acetone:ethanol) were used

successively on the whole of the operative site. The pregnant uterus was exposed by an oblique abdominal incision and the main umbilical arterial branches and venous tributaries of cotyledons at each end of the fetus located by palpation.

Vinyl tubing (0.5 mm I.D. 0.9 mm O.D. or 0.4 mm I.D. 0.8 mm O.D.) attached to 60 - 85 cm of larger diameter vinyl tubing (0.63 mm I.D. 1.4 mm O.D.) was threaded into a small tributary and secured so that its tip lay about 5 cm inside the major vessels. A retaining cuff of vinyl tubing (1.4 mm I.D. 1.9 mm O.D.) was placed over the junction of the two types of tubing. To the free end of the larger diameter tubing a three-way luer tap was attached.

The uterus was replaced in the abdominal cavity and the incision closed with catgut sutures. 3 - 5 cm of the catheters were left exposed by dorsally extending the anterior end of the skin incision by about 8 cm and closing this over the catheters with silk sutures. To protect the catheters a large bandage of two thicknesses of lint was secured over the animal's back with strips of 7.5 cm bandage, so that most of the ventral and lateral abdomen was covered.

After surgery the sheep remained in a heated room (22 - 25^{o}C) for 2 - 3 days. After regaining consciousness, chopped hay and grass were fed for the first 9 hours and thereafter pelleted food. For 1 - 2 days 0.9 per cent (w/v) saline was given in place of water to help restore fluid and electrolyte balance. 20 - 30 mg/kg procaine penicillin with 20 - 25 mg/kg dihydrostreptomycin or 5 - 7 mg/kg oxytetracycline hydrochloride were given for 3 days and skin sutures removed after 2 weeks. Fetal blood was sampled daily to monitor the condition and to keep the catheters patent. (For detailed discussion see Page 71).

Experimental Conditions

The experiments in all but one acute study involved continuous infusion of radioactive material for four or ten hours. During this time the sheep was kept in the pen to which it was accustomed and a dividing barrier was placed across the pen so that the animal could lie or stand but not turn round. The sheep had been similarly restricted in the preoperative conditioning period.
Precalibrated syringe infusion pumps were placed on a shelf above the animal. An infusion line (7 - 10 m vinyl tubing 0.9 I.D. 1.5 O.D. Portex Ltd.) sterilized by rinsing with 0.1 per cent w/v thiomersal solution (50:50 v/v acetone: ethanol) was attached to the syringe containing the radioactive tracer solution. Residual thiomersal solution and air were expelled from the line with sterile isotonic saline solution before attaching to the vascular catheter.

In fetal experiments both umbilical venous and arterial routes were used for infusions, the route depending upon the ease with which fetal blood could be withdrawn. Preferentially the umbilical venous route was chosen (this being the route by which nutrient substances are transferred from the mother). If blood sampling was difficult from the arterial catheter, the infusion was given into the artery and the samples obtained from the umbilical vein. Experiments in which saline or insulin were infused simultaneously with the radioactive tracer amino acid had a Y junction (Portex) to connect the two syringe infusion lines positioned about half-way between syringe and entry into the umbilical catheter.

In maternal infusion experiments both left and right jugular veins were cannulated, samples being withdrawn from one and radioactive material being infused into the other. Only one jugular vein was cannulated if infusion was into the fetus.

At the end of the syringe stroke the syringe was filled with isotonic saline and infusion continued until the dead space between the syringe and umbilical catheter had been cleared.

At predetermined intervals during the infusion, maternal and fetal blood samples were obtained by the following techniques:-

Maternal Blood Sampling: After removing the catheter plug drops of free flowing blood were discarded to clear the cannula of any static blood. A sterile 10 ml syringe (Plastipak, Becton Dickinson & Co., Ireland) was filled with the maternal jugular vein sample. The cannula was flushed with a small quantity of heparinised saline and the cap replaced.

Fetal Blood Sampling: All syringes (5 ml Plastipak) were individually packed in autoclavable nylon bags and sterilised in an autoclave before use. 0.1 per cent thiomersal solution (50:50 w/v acetone: ethanol) was used to drench the catheters and surrounding skin and the following technique adopted to prevent contamination of fetal samples:-

The catheter was flushed with 0.5 - 1 ml isotonic saline (volume of catheter and tap 0.5 - 0.7 ml) and 0.75 ml of fetal blood withdrawn into the same syringe and the syringe discarded. The fetal blood sample, usually 2-3 ml in volume, was withdrawn slowly into a fresh syringe containing o.25 mg dry heparin. 3.0 ml isotonic w/v saline was flushed through the catheter followed by 0.75 - 1.0 heparinised saline solution (3 mg heparin/100 ml saline solution). Thiomersal was again used to wash the catheters and skin before replacing the bandages securing the catheters.

Termination of Experiment

After the final samples had been collected, nembutal was administered via the maternal jugular cannula. Approximately 300 ml of maternal blood was taken and mixed with 15,000 units of heparin in a 500 ml bottle. The sheep was removed from the pen and immediately the animal was unconscious the abdomen was opened and the uterus dissected. The position of the catheter tips in the fetal vessels was determined and approximately 100 ml blood was withdrawn from the fetal catheter and placed into a 500 ml bottle containing 5,000 units heparin. Amniotic and allantoic fluids were sampled by withdrawing fluid into 50 ml syringes and capping these. Placental cotyledons were collected in polythene bags. The lamb or lambs were freed from the surrounding membranes and weighed in a plastic bag. Samples of fetal tissue were removed by dissection and where possible fetal urine was obtained by bladder puncture. Duplicate tissue samples were placed in universal containers or pots, the sample for autoradiography being place in 1.5 per cent glutaraldehyde as fixative. All tissues and samples were taken to the Paediatric Laboratory, Simpson Memorial Maternity Pavilion, where the terminal blood samples from mother and fetus, and amniotic and allantoic fluids were centrifuged. Plasma was collected from the blood and any extraneous matter was removed from the amniotic and allantoic fluids. All tissues and samples were stored at $-70^{o}C$ until analyses were carried out.

PRELIMINARY TREATMENT OF BLOOD SAMPLES

a. Maternal

Of the 10 ml blood taken, approximately 8 ml was expelled from the syringe into a 10 ml lithium heparin tube (Stayne Laboratories Ltd.) for separation into plasma and red blood cells. 2 ml was placed in a 2 ml lithium heparin tube for whole blood analysis, haematocrit and haemoglobin determinations and 0.5 - 1 ml into sodium fluoride tubes for blood glucose determination when this was indicated in the experimental procedure. All samples were well mixed in the tube to prevent clotting. Plasma was separated from the blood by centrifugation as described for the fetal samples.

b. Fetal

1 ml of fetal blood was expelled into a 2 ml lithium heparin tube and 0.5 - 1 ml into a blood glucose tube as for the maternal samples. The remainder was immediately centrifuged; in years 1 and 2 in an MSE bench centrifuge at 2000 rpm for 15 minutes, and in year 3 in 2 ml polythylene tubes in a Beckman Microfuge at 13,000 rpm for five minutes.

The separated plasma was initially stored at -20°C and the RBCS, whole blood and blood glucose tubes were stored at 4°C. After completion of the experiment, or the evening of the infusion, whichever was the sooner, samples were taken to the Simpson Memorial Maternity Pavilion where plasma was stored at -70°C and the other samples at 4°C until analysed.

TABLE III

Physical Details of Sheep and Route of Infusion Employed

Experiment	Sheep No.	Weight kg	No. of fetuses	Condition	Gest. days	Days post operation	Route of Infusion
T1	H 317	62.0	2	a. live b. live	140	58	mv
T2	H 317	49.7	2	a. live b. live	90	8	mv
T3	H 354	56.9	2	a. live b. live	135	25	mv
T4	H 351	49.4	2	a. dead b. live	131	26	uv

mv = Maternal vein (jugular)

uv = Umbilical vein

gest. = gestation

PART II

Tritium Labelled Amino Acid Infusions

Tritiated phenylalanine was employed both to establish that 250 μCi infused for 4 hours at 50 μCi/hour after an initial bolus of 50 μCi would produce measurable activity in both fetal and maternal plasma, and that activity would reach a plateau in plasma water within the time of infusion.

Two preliminary maternal infusions were undertaken, but only one provided fetal samples. In a single fetal infusion, maternal samples were continually available, and fetal samples from day 1. A fourth experiment to study the distribution of activity following a bolus injection was carried out.

L Phenyl (2,3 - ^{3}H) alanine from the Radiochemical Centre, Amersham was used in all experiments. This amino acid had a specific activity of 15.6 Ci/mmol and a radioactive concentration of 1mCi/ml.

None of the animals were sacrificed so tissue was not available for autoradiography or total tissue counting. Table III gives details of the animals used in the experiments and the routes of infusion.

As the fetal weight was unknown no estimate of the total activity in fetal plasma could be made. Also, as few measurements of S.A were made, and there appeared to be both a large contribution to plasma water activity from sources other than the infused amino acid and loss of activity on amino acid incorporation into protein, flux and incorporation rates were not estimated.

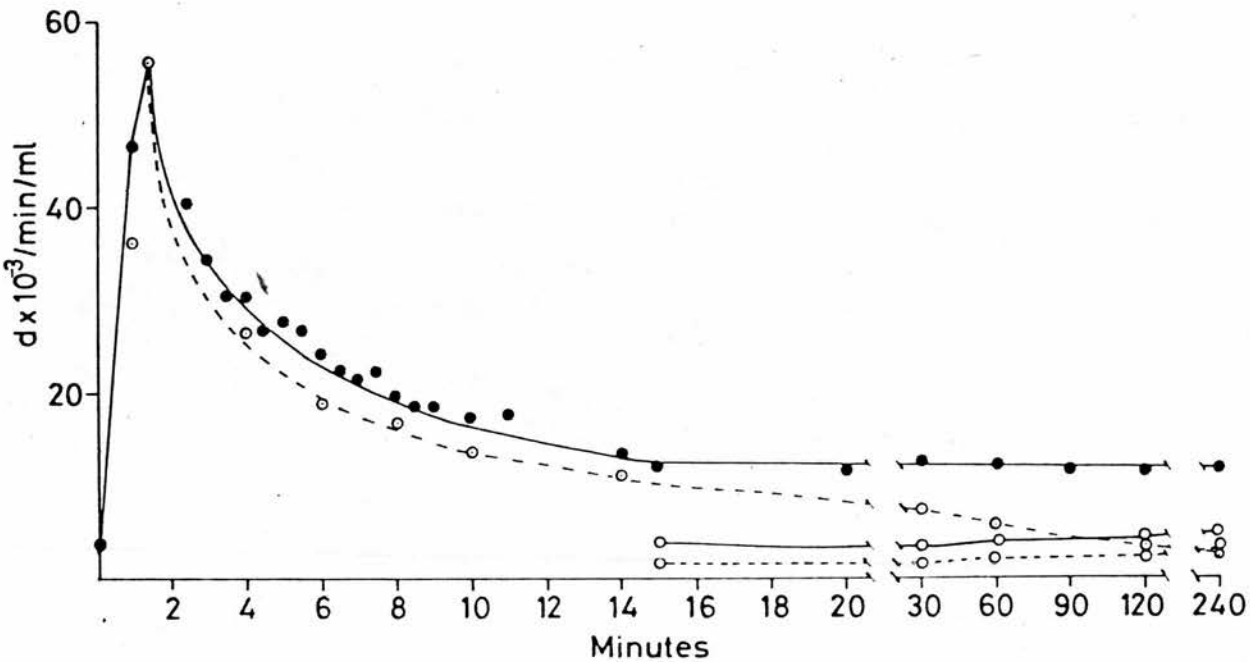


Fig 1i

Activity in maternal (●-) and fetal (O-) total plasma* and maternal (0---) and fetal (O---) deproteinised plasma* for 4 hours after a 250 μ Ci L ^{3}H 2 - 3 phenylalanine bolus injection into the maternal jugular vein.

* In all graphs

total plasma represents radioactivity present in plasma protein and plasma water.

deproteinised plasma represents radioactivity present in plasma water alone.

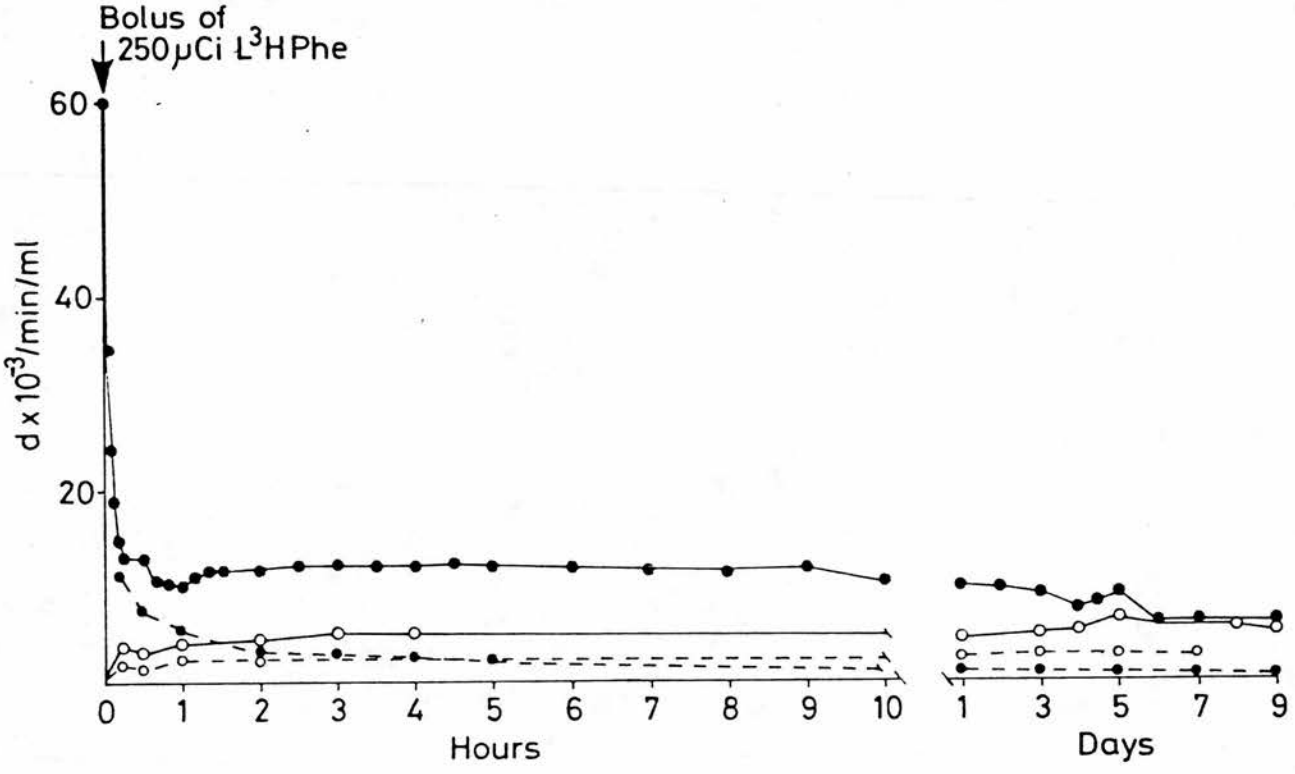


Fig 1ii

Activity in maternal (●–) and fetal (○–) total plasma and maternal (●---) and fetal (○---) deproteinised plasma following a 250 μ Ci L^3H 2 - 3 phenylalanine bolus injection into the maternal jugular vein.

a. Bolus Injection into Maternal Vein

Experiment T1

Experimental Details

L^3H Phenylalanine 250 μ Ci in 5 ml of isotonic saline injected within 15 seconds through a right superior vena caval cannula inserted into the right atrium. After one minute the first active maternal blood sample was taken from the left maternal jugular vein at 08.01 hours. Maternal samples were taken at 30 second intervals for 10 minutes and at one minute intervals between 10 and 15 minutes post injection. Samples were taken at 20, 25 and 30 minutes and then at 10 minute intervals for the next hour (1½ hours post injection). Half hourly samples were taken until 5 hours post injection and hourly samples from 5 to 10 hours. A 12 hour and 16 hour (24.00 hours) sample were taken on the day of the experiment and 12 hourly samples from 25 hours (i.e. 09.00 hours the following day) until 72 hours post infusion. The sampling was continued on a daily basis for a further 3 days.

Fetal samples from fetus B were taken from the umbilical arterial catheter until 4 days post injection, when umbilical venous samples were obtained. The first fetal sample radioactive was taken at 15 minutes, the second at 30 minutes and then at 1,2,4,8,16 and 25 hours after the injection and daily at 09.00 hours until the end of the experiment.

Results

Total radioactivity in plasma and plasma water during the first 240 minutes following the 250 uCi L^3H phenylalanine injection is shown in Fig 1i and during the 9 days of study in Fig 1ii.

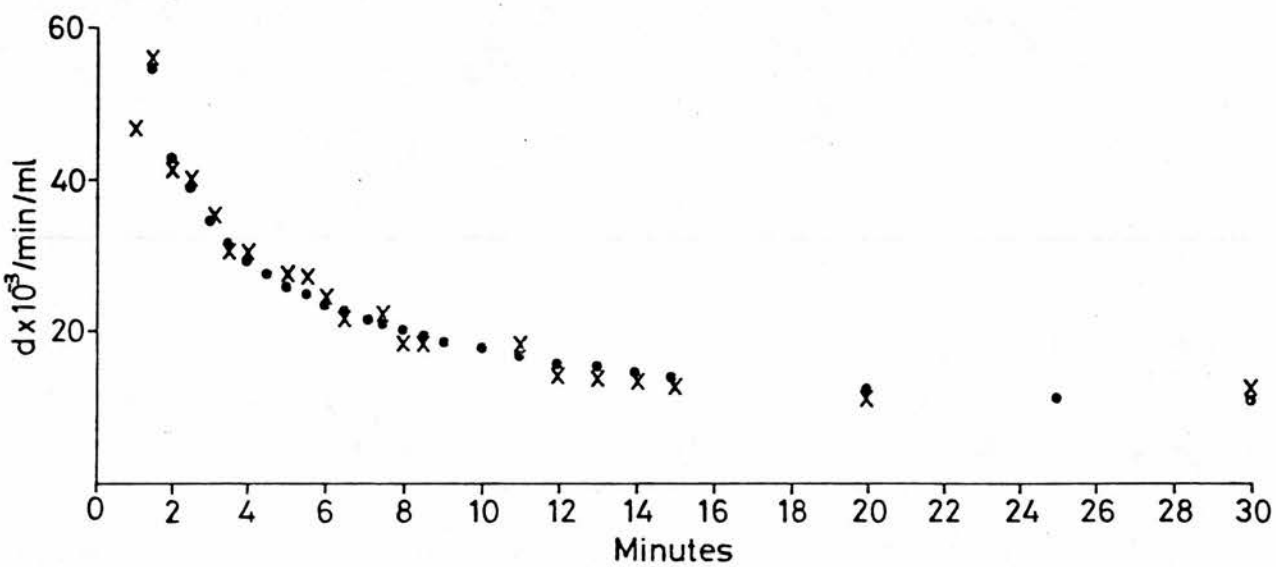


Fig liii

Calculated activity (●) from the formula :- $Y = X_1 + X_2 e^{-\lambda_2 t} + X_3 e^{-\lambda_3 t}$

where $X_1 = 10{,}561 \pm 736$

$X_2 = 29{,}101 \pm 3171$

$X_3 = 8830 \pm 515$

$\lambda_2 = 0.140 \pm 0.017$

$\lambda_3 = 0.948 \pm 0.114$

and measured activity (x) in maternal plasma after a bolus injection of 250 μ Ci L^3H 2 - 3 phenylalanine.

Compartmental Analysis

A computer was employed to examine the decay curve of radioactivity in an attempt to assess the number of pools of aminoacid with which the injected material may mix.

The best mathematical "fit" to the curve was obtained from the equation $X_1 + X_2 e^{-\lambda_2 t} + X_3 e^{-\lambda_3 t}$ (i.e. 2 exponentials plus a constant). Values for X and λ and a plot of this calculated curve together with the measured curve are shown in Fig iii. This could represent constant metabolism of phenylalanine (X_1) coupled with rapid but decreasing removal from the plasma to maternal tissue cells (X_2) and slower removal to the fetus (X_3). Other formula fitted to the curve included $X + X_2 e^{-\lambda_2 t}$, $X_1 e^{-\lambda_1 t} + X_2 e^{-\lambda_2 t}$ and $X_1 e^{-\lambda_1 t} + X_2 e^{-\lambda_2 t} + X_3 e^{-\lambda_3 t}$

Discussion

From experiment T1, tritiated phenylalanine, injected as a bolus into the maternal circulation appears to equilibrate rapidly (Fig i and ii). A plateau concentration (12.00-13.00 X 10^3d/min/ml) of total activity in maternal plasma was reached within 12 minutes. During this time 90 per cent of the label was found in the supernatant of the protein precipitated plasma i.e. plasma water, probably as the free amino acid. Although a constant level of total radioactivity was maintained for six hours, one hour post injection the proportion of activity present in the supernatant had declined to less than 50 per cent. After six hours it was less than 15 per cent. Thus the proportion of bound activity increased whilst activity associated with the free amino acid declined. From six hours, total radioactivity also diminished.

During the initial mixing phase transfer of the amino acid to the fetus occurred, the 15 minute fetal plasma sample having an activity of 3.80 X 10^3d/min/ml. Little further transport occurred after this, probably a result of the fall in maternal concentration of labelled free plasma water amino acids - maximal fetal values being found at three hours(5.2 X 10^3d/min/ml). The proportion of bound radioactivity remained constant at approximately 50 per cent of the total activity. From four hours fetal plasma water had a slightly higher activity (2.00 X 10^3d/min/ml) than maternal plasma water (2.00 X 10^3d/min/ml decreasing to 0.50 X 10^3d/min/ml) reflecting equilibration of free amino acid between and within both maternal and fetal plasma free amino acid pools, fetal plasma amino acids being at a higher concentration than those of maternal plasma. Maternal total activity decreased slowly whereas fetal total activity slowly increased, until by day six both had similar activity - 5.80 X 10^3d/min/ml. From these results a 250 μCi bolus injection would not be expected to influence a constant infusion experiment beyond two hours. If the bolus injection were smaller and the subsequent infusion was at a rate appreciably in excess of that of the decreasing activity from the bolus injection, the time to reach equilibrium should be less.

b. Infusions

i Maternal Infusions

Experimental Details

Experiment T2

Maternal Infusion L^3H Phenylalanine

L^3H Phenylalanine 250 μ Ci was diluted to 50 ml with sterile isotonic saline.

At zero time minus 15 minutes baseline maternal samples were taken and at zero time minus 5 minutes (14.45 hours) a bolus of 10 ml tritiated phenylalanine solution was injected. Infusion at a rate of 10 ml (50 μ Ci/hour) into the maternal jugular vein was commenced at 14.50 hours and continued for four hours. Maternal samples were taken at 30 minute intervals over the first four hours and then at 7, 9 and 19¼ hours after the bolus injection. Fetal samples were not available.

Experiment T3

Maternal Infusion L^3H Phenylalanine

L^3H Phenylalanine 250 μ Ci in 50 mls isotonic saline was infused into the maternal venous catheter after both maternal and fetal baseline samples had been taken. A bolus of 10 ml was injected between 14.23 and 14.30 hours and thereafter a continuous infusion of 50 μ Ci -(10 ml/hour) was continued until 18.32 hours. Blood samples were collected at 30 minute intervals throughout the infusion. At 19.12 hours (4 hours 42 minutes after the start of infusion) an allantoic fluid sample was obtained and at 4¾ and 8 hours post infusion maternal and fetal samples were taken. Thereafter, maternal, fetal and allantoic fluid samples were taken at approximately 09.00 hours during the next four days.

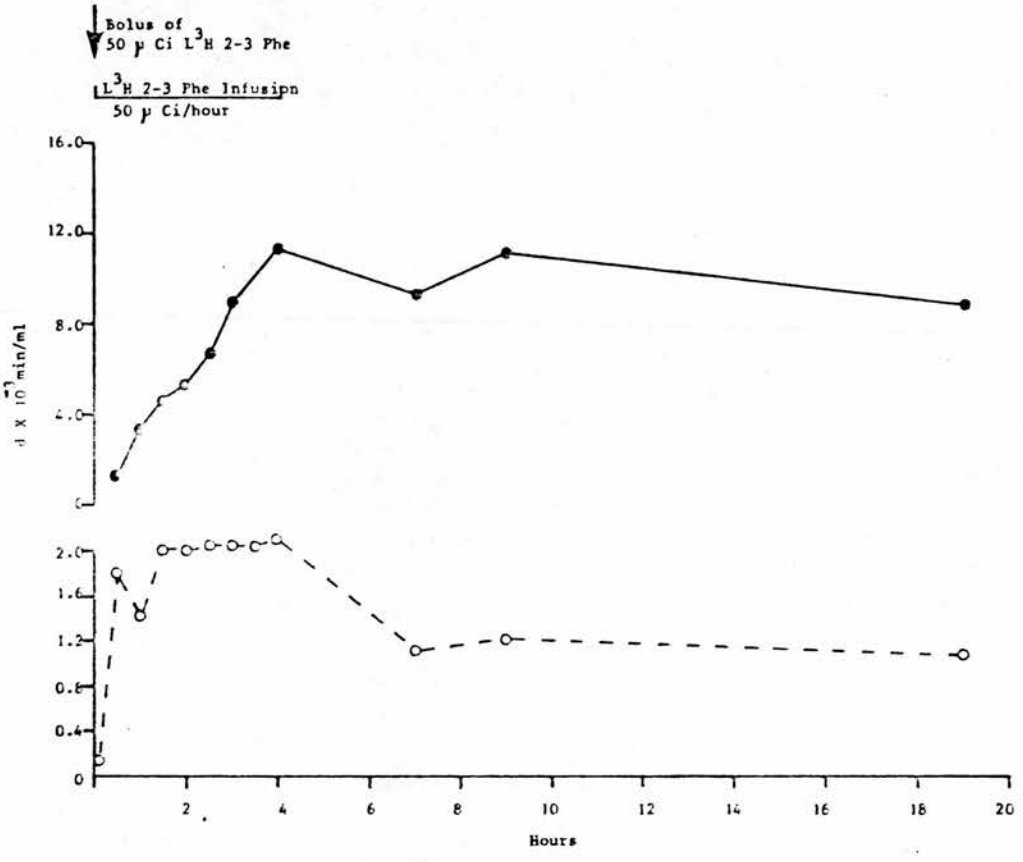


Fig 2.

Activity in maternal total (●–) and deproteinised plasma (O---) during and after infusion of 250 μCi L^3H 2 - 3 phenylalanine into the maternal jugular vein.

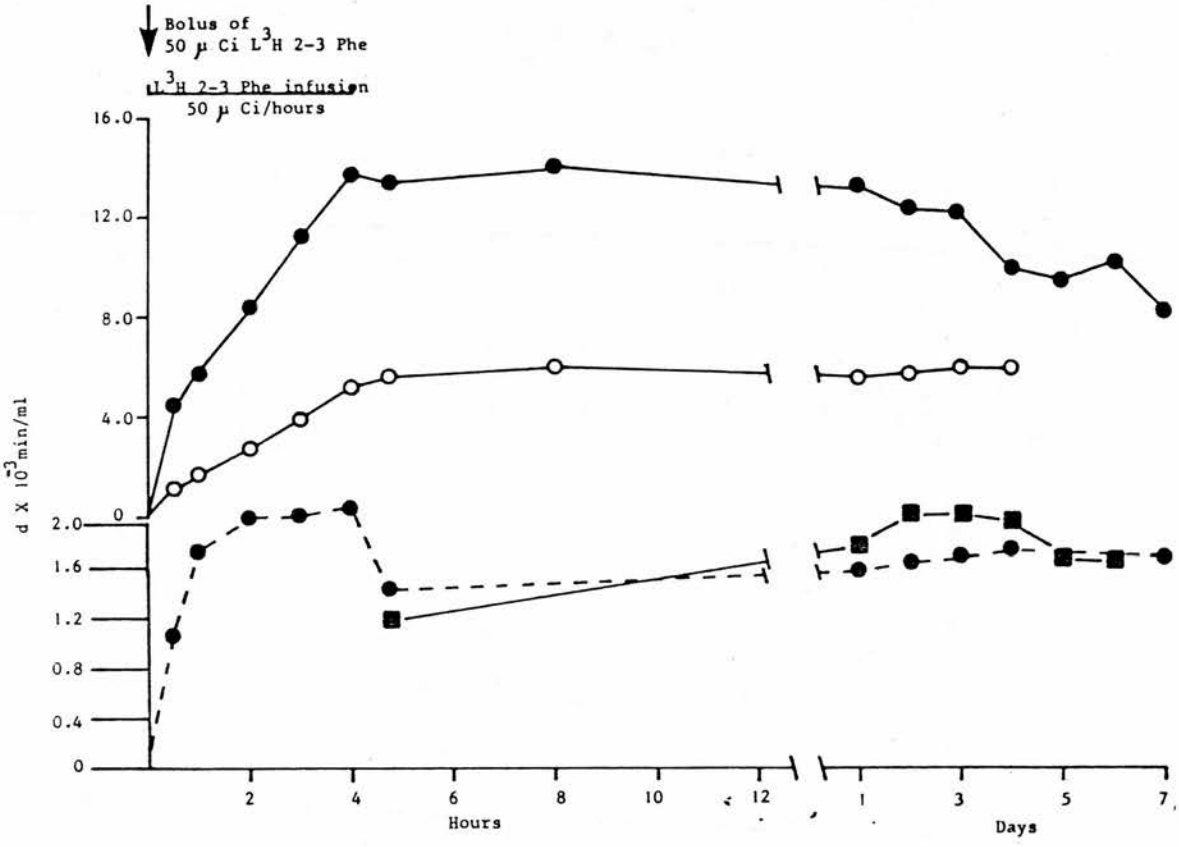


Fig 3

Activity in maternal (●) and fetal (O) total plasma and in maternal deproteinised plasma (---) during and after infusion of 250 μ Ci $L^{3}H$ 2 - 3 phenylalanine into the maternal vein. Amniotic fluid activity (■) was measured from 4¾ hours to day 6.

Results

Infusion of L^{3}H phenylalanine into the maternal jugular vein - experiment T2 (Fig 2) and experiment T3 (Fig 3) resulted in an initial rapid increase in radioactivity in both maternal plasma water and total plasma fractions. However, although it appears possible to infuse amino acids at a rate sufficient to permit equilibration of the plasma water free amino acid pool after 1½ hours, the total (i.e. protein/peptide) pool is clearly not saturated and incorporation of labelled amino acid into protein or peptide is occurring at a near linear rate throughout the infusion period, activity increasing at 3.01×10^3 d/min/ml/hr in experiment T2 and 3.02×10^3 d/min/ml/hr in experiment T3.

In experiment T3 thirty minutes after starting infusion total plasma activity of the fetus already exceeded that of maternal deproteinised plasma, demonstrating rapid transfer. Fetal total activity continued increasing at 1.12×10^3 d/min/ml/hr during the infusion period.

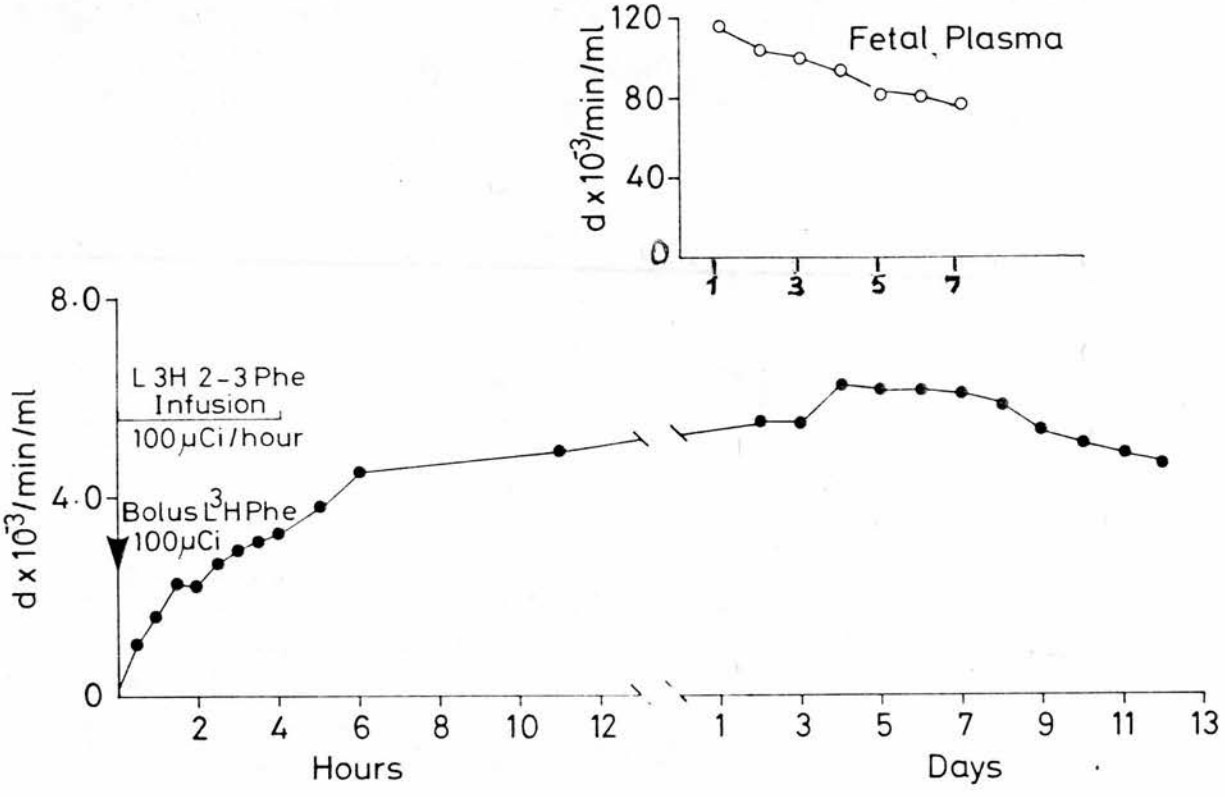


Fig 4

Activity in maternal total plasma (●-) during and after 500 µ Ci $L^{3}H$ 2 - 3 phenylalanine infusion into the umbilical vein and in fetal total plasma (0-) 1 to 7 days after the infusion.

ii Fetal Infusion

Experiment T4

Fetal Infusion L^3H Phenylalanine

Experimental Details

L^3H Phenylalanine 500 μ Ci in 50 ml sterile isotonic saline were infused into the fetal vein. A baseline maternal blood sample was taken and a 10 ml bolus injection into the fetal venous catheter given over 4 minutes (from 09.26 - 09.30 hours). Infusion at 10 ml (100 μ Ci/hour) was commenced and continued for four hours. Samples were taken at 30 minute intervals for 5 hours and thereafter at 11½ hours and daily at 09.00 hours for four days. Fetal samples were only obtained from 24 hours onwards.

Results

Retrograde amino acid transport from fetal to maternal circulation was demonstrated in experiment T4 (Fig 4). This "back" transfer, detected by increasing radioactivity in maternal total plasma samples, continued at approximately the same rate 0.60 X 10^3d/min/ml between 0 and 4 hours and 0.54 X 10^3d/min/ml between 2 and 4 hours and for two hours beyond the end of the infusion probably because relatively high concentrations of labelled amino acids are maintained in the fetal plasma relative to those of maternal plasma - (S.A. fetal plasma would exceed that of maternal plasma.)

Discussion

Maternal and Fetal ^{3}H Infusion

The bolus injection (50 μ Ci) obviously accounted for a large proportion of the early increase in activity with, as in experiment T4, approximately 50 per cent of the total activity being in plasma water at 30 minutes. The decrease in plasma water activity at one hour could be due to equilibration between the decreasing contribution of activity from the bolus injection and the increasing contribution from the constantly infused phenylalanine, but could also be caused by changes in the metabolic equilibrium (page 239).

In both maternal infusions there was a rapid decrease of radioactivity in plasma water and total radioactivity ceased to increase when the infusion stopped. In experiment T3, the increase in fetal total radioactivity was also reduced at this time, suggesting transfer of labelled amino acid decreased. This is in contrast to experiment T4, fetal infusion, in which reverse transfer from fetus to mother would appear to continue, probably a result of diffusion from the higher concentration fluid (fetal plasma) to the maternal environment of lower concentration and radioactivity.

Activity measurements in allantoic fluid in experiment T3 would indicate that although transfer occurs into this compartment, equilibration is slow - activity in maternal and fetal plasma being relatively stable from four hours onwards but allantoic fluid activity increasing until day 2. However, a decreasing fluid volume would have the same effect and S A. measurement (requiring a higher level of activity) would be necessary to establish the time to equilibration.

From these experiments the following conclusions can be drawn:

1. 250 μ Ci is sufficient to give measurable activity in fetal plasma and to demonstrate "back" diffusion to maternal plasma, although levels are low for S.A. measurement.
2. A bolus injection of 50 μ Ci does not have any influence on results after 1½ hours.
3. Plateau activity is found in the plasma water fraction of the infused animal - i.e. ewe or fetus, from two hours after the bolus injection until the end of infusion.
4. Deproteinized fetal plasma has a higher concentration of labelled amino acids than the deproteinized maternal plasma.
5. The increase in protein bound activity is linear throughout the experiments.
6. The experimental design produced no problems in the management of the sheep or sampling from the implanted catheters.

TABLE IV

Physical Details of Sheep and Route of Infusion Employed

Experiment	Amino Acid	Sheep No.	No. of fetuses	Condi-tions	Weight kg	Gest. Days	Days post operative	Route of Infusion
C1	^{14}C Phe	H 263	1	live	4.28	126	20	mv
C2	^{14}C Leu	H 343	1	live	4.05	127	16	mv
C3	^{14}C Phe	H 330	1	live	4.07	130	15	uv
C4	^{14}C Phe	H 285	1	live	3.98	126	16	mv
	^{3}H Phe							uv

Phe = phenylalanine

Leu = leucine

Gest = gestation

Part III

$L^{14}C$ Amino Acid Infusions

Maternal and fetal infusions similar to those of Year One were repeated using $L^{14}C$ phenylalanine. Additionally a simultaneous infusion of $L^{14}C$ into the mother and $L^{3}H$ phenylalanine into the fetus was carried out. Infusion of $L^{14}C$ leucine into the maternal jugular vein was also studied.

In addition to measurements of radioactivity in total plasma, plasma water and blood, S A was determined in plasma water of the phenylalanine maternal, fetal and dual infusions. Plasma protein was hydrolysed in selected samples of the phenylalanine maternal and dual infusions. Measurements of radioactivity in those hydrolysed samples are presented together in the last part of this section.

L (U - ^{14}C) Phenylalanine specific activity 522 mCi/mmol with a radioactive concentration of 50 μCi/ml was used in all phenylalanine experiments.

L (U - ^{14}C) Leucine, specific activity 324 m Ci/mmol with a radioactive concentration of 50 μ Ci/ml was used in experiment 3. Labelled amino acids were obtained from Radiochemical Centre, Amersham. Tritiated L phenylalanine was prepared as described in Part 1.

Table IV gives details of the animals used in the experiments and the type and routes of infusion.

At the end of the experiment the animals were sacrificed. Allantoic and amniotic fluids were obtained, blood films of maternal and fetal blood made and the following tissues taken from the lamb:- thymus, thyroid, lung, heart, liver, spleen, adrenal, kidney, muscle, brain, placental cotyledons. Fetal urine was obtained when present.

i Maternal ^{14}C Infusions

Experimental Details

Experiment C_1

Maternal Infusion LU^{14}C Phenylalanine

^{14}C L Phenylalanine 250 μ Ci in 25 ml of isotonic saline was infused into the maternal jugular vein by syringe pump at 5 ml (50 μ Ci/hour) for four hours. A bolus of 5 ml was given from the syringe immediately prior to commencement of infusion at 11.15 hours. Maternal samples were taken from the other jugular vein and fetal samples via the umbilical vein catheter. Maternal and fetal blood samples were taken at ½, 1, 1½. 2, 3, 4, 5, 6 and 11 hours and daily at 09.00 hours for a further nine days. In addition maternal samples, but not fetal samples were taken at 2½, 3½, and 8 hours. At sacrifice fetal urine was not obtained.

Experiment C2

Maternal Infusion LU^{14}C Leucine

LU - ^{14}C Leucine 250 μ Ci in 25 mls isotonic saline was infused and samples collected as in experiment C1 The infusion was commenced at 11.10 hours after a bolus injection of 5 ml. An additional blood sample from both mother and fetus was taken at 15 minutes, and the sample equivalent to the 11 hour sample was taken at 10 hours. The animal was sacrificed after 7 days. Fetal urine was obtained.

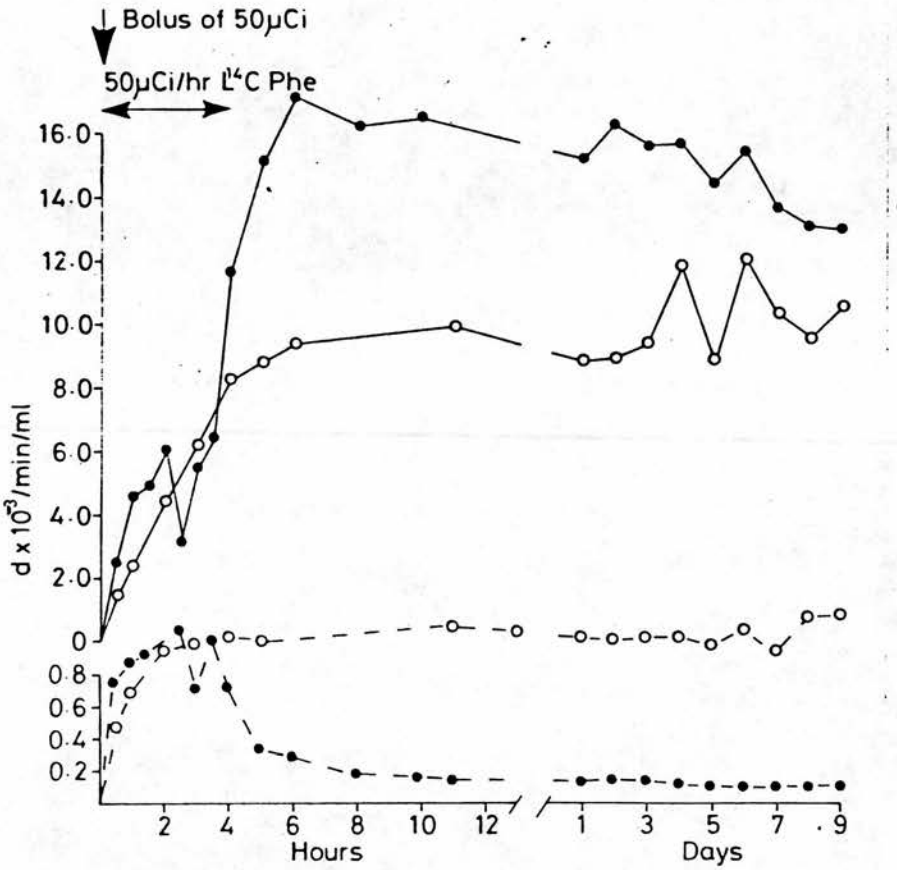


Fig 5i

Maternal (●) and fetal (0) plasma total (-) and deproteinised (---) activity during and after 250 μ Ci L ^{14}C phenylalanine infusion into the maternal jugular vein.

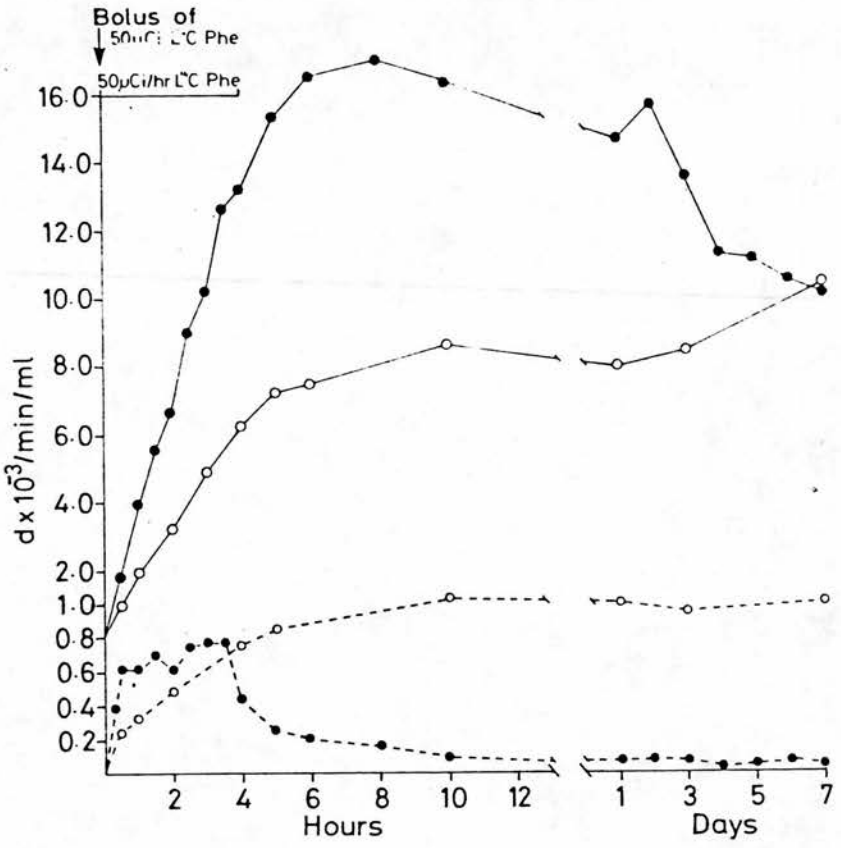


Fig 6i

Maternal (●) and fetal (0) plasma total (-) and deproteinised (---) activities during and after 250 µ Ci L ^{14}C leucine infusion into the maternal jugular vein.

Results

a. Haematocrits

Haematocrits in both fetal and maternal blood of experiment C1 remained reasonably constant throughout the experimental period. Maternal range was from 25-27.5% and fetal from 32-36.5%.

In experiment C2 maternal haematocrits remained very stable (28-29%) during the first 24 hours and in days 2-7 ranged between 24.5 and 29%. Fetal haematocrits also remained constant (35-37%) up to day 3, but had increased to 44% on day 7. Individual haematocrit values may be found in Appendix 6.

b. Total Plasma Radioactivity Measurements

Maternal total radioactivity increase was linear in both experiments (fig 5i and 6i). The only exception was the decrease in total activity between hours 2 and 3 of the phenylalanine infusion, mirrored in deproteinised plasma 30 minutes later which could be due to changing fluid volumes, haematocrit values, placental transfer or variations in the infusion rate (viz. p222).

The increase in total fetal plasma radioactivity was also linear, but the rate of increase (1.94×10^3d/min/ml/hr and 1.51×10^3d/min/ml/hr for experiments C1 and C2 respectively) was slower than that of maternal total plasma in both experiments.

c. Deproteinised Radioactivity Measurement

In both experiments (Fig 5i and 6i) the increase and decrease in maternal deproteinised (i.e. plasma water) radioactivity on starting and stopping infusion respectively was rapid. There was only a small increase in the activity of deproteinised plasma in maternal samples taken between 30 minutes and 4 hours (final) after the start of infusion. In both experiments there was a rapid decrease from the plateau activity in the hour after completing the infusion, with maternal deproteinised plasma radioactivity being below that of the fetus in samples taken after ending the infusion. Fetal deproteinised plasma activities differed from those of maternal samples in that the increase was slower on infusing the amino acid and there was no decrease on ending it.

The two infusions differed in that deproteinised radioactivity reached a plateau, similar to that of maternal samples in the phenylalanine C1 infusion, but continued to increase in the leucine C2 infusion. The final radioactivity was however also similar to that of maternal samples at the end of the infusion.

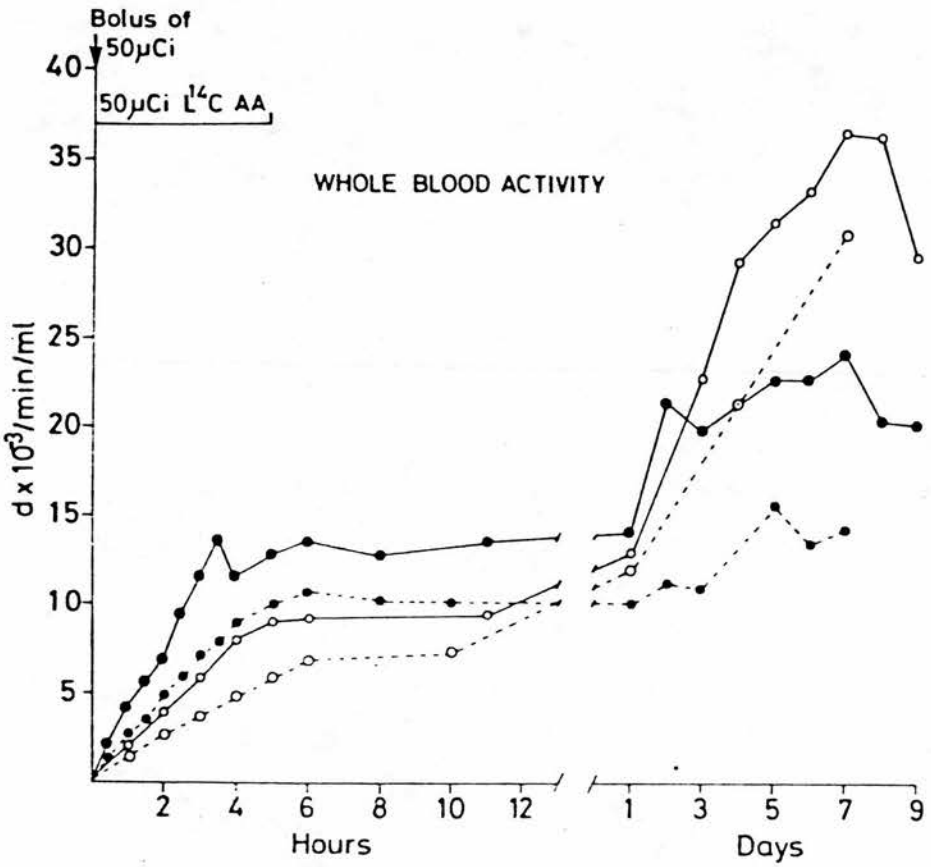


Fig 5ii

Maternal (●) and fetal (O) activity in whole blood during and after 250 u Ci $L^{14}C$ phenylalanine (-) and 250 μ Ci $L^{14}C$ leucine (---) infusions into the maternal jugular vein.

d. Radioactivity in Whole Blood

During the first 24 hours radioactivity in whole blood (fig 6ii) was similar to that of total plasma activity, although in experiment C1 (phenylalanine infusion) the slight fall found in maternal plasma at 2½ hours did not appear to be present. This might indicate a sudden increase in the proportion of activity present in or attached to the blood cells as an increase in haematocrit was not observed. An increase with time was observed in the cellular proportion of radioactivity, and at all times a higher percentage of activity was associated with fetal RBC than was found in maternal RBC's. The proportion in maternal samples increased from approximately 12% to 45-60% between the end of infusion and the samples taken approximately one week later (5-9 days). Similarly in fetal samples there was an increase from 35-55% to 75-80%.

e. Tissue Digestion Allantoic and Amniotic Fluids Radioactivity

Details of radioactivity found in tissues are given on page 190. In the phenylalanine experiment total activities were 0.44 x 10^3d/min/ml in the allantoic and 0.19 x 10^3d/min/ml in the amniotic fluid sampled at delivery, and in the leucine experiment the radioactivities were 0.61 x 10^3d/min/ml and 0.23 x 10^3d/min/ml in allantoic and amniotic fluids respectively.

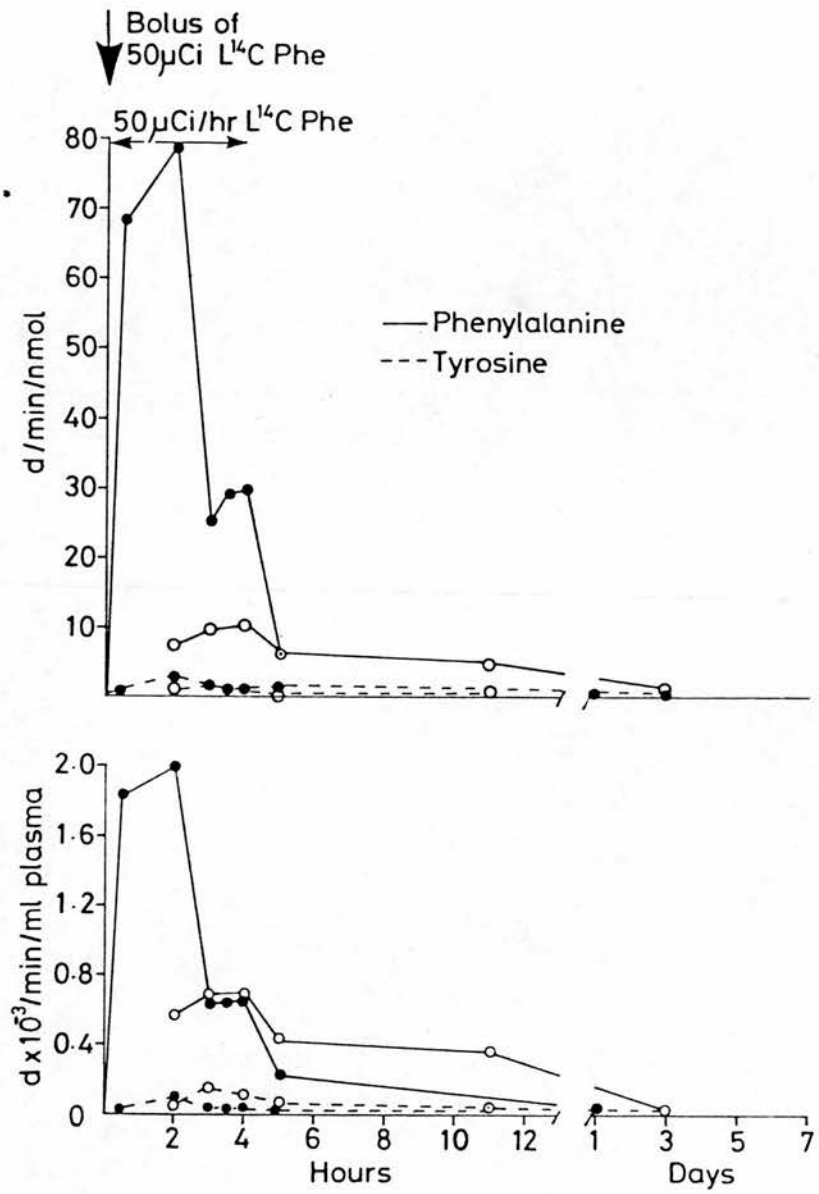


Fig 5ii

Maternal (●) and fetal (0) phenylalanine (-) and tyrosine (---) activity in plasma water (below) and free amino acid SA (above) during and after 250 μ Ci $L^{14}C$ phenylalanine infusion into the maternal jugular vein.

TABLE V

Amino Acids Concentrations with Time

	Time (hr)	Tyrosine µmol/l	Phenylalanine µmol/l
Maternal	½	41.5	26.9
	2	32.1	25.3
	3	35.9	26.5
	3½	34.2	24.0
	4	30.3	23.9
	5	33.1	32.2
	22	64.0	44.3
	3 day	29.2	22.1
	7 day	47.0	37.0
Fetal	2	93.2	79.2
	3	97.0	75.4
	4	81.2	64.4
	5	88.8	71.0
	11	109.2	77.0
	3 days	215.9	217.0

f. Experiment C1 -(Maternal Phenylalanine Infusion)

Specific Activity Measurement

Plasma water specific activity was determined in maternal samples taken at 30 min, 2, 3, 3½, 4 and 22 hours and 3 and 7 days and in fetal samples taken at 2, 3, 4, 5 and 11 hours and day 3. Both plasma water and hydrolysate specific activities were determined for phenylalanine and tyrosine on the 2, 4 and 22 hour, 3 and 7 days maternal samples. Hydrolysate results have been combined with those of the dual infusion and may be found on page 126.

The specific activity of free plasma phenylalanine and tyrosine is shown in fig 5ii . Changes in free phenylalanine and tyrosine plasma concentration may be seen from Table V.

Maternal Values

Analysis of the first maternal sample at 30 minutes showed that the bolus injection of 50 μ Ci phenylalanine had produced a rapid rise in SA reaching 68.20 d/min/nmol and that ^{14}C labelled tyrosine was already present (0.52 d/min/nmol) thus indicating that rapid hydroxylation of the phenylalanine must have occurred.

The tyrosine:phenylalanine SA ratio rose until the 22 hours sample although individual activities ranged from 0.98 to 78.90 d/min/nmol phenylalanine during this time. At the end of infusion a rapid fall in phenylalanine but not tyrosine specific activity was observed. However, activity was detectable in the phenylalanine fraction up to day 7 but not beyond 22 hours in the tyrosine fraction.

From the plateau SA maternal flux was calculated to be 4036 μmol/hr.

Fetal

Fetal plasma water phenylalanine and tyrosine specific activities were below maternal values, this being largely a result of the greater concentration of amino acids in fetal plasma, the activity per millilitre of plasma at equilibrium being similar in both maternal and fetal samples. Fetal phenylalanine SA increased from 7.03 d/min/nmol at 2 hours to 9.20 at 3 hours then only slowly to 10.20 d/min/nmol at 4 hours, the plasma water phenylalanine concentration thereafter falling from 75.4 to 64.4 μmol/l. This decrease in fetal plasma water phenylalanine concentration was less marked than that found in maternal plasma and significant quantities of labelled phenylalanine and tyrosine were found in plasma 7 hours after the infusion had stopped. Tyrosine SA equilibrated at the same time as phenylalanine SA i.e. from 3 hours onwards although during this time plasma amino acid concentration also fell from 97 to 81.2 μmol/l. The ratio of tyrosine to phenylalanine SA reached a maximum of 0.15 at 3 hours, and unlike maternal plasma little change was noted thereafter.

g Distribution of Free and Bound Radioactivity

The maximum percentage of the ^{14}C phenylalanine infused recorded in the maternal plasma as phenylalanine and tyrosine is 6.89 per cent at 22 hours, the major fraction of this being present in the hydrolysate - 6.85 per cent of the infused radioactivity. The maximum percentage recorded as total radioactivity was 8.0 per cent at 6 hours, followed by a plateau of 7 per cent at sacrifice. The only sample with a greater percentage in the plasma water was at 30 minutes - plasma water 2.85 per cent with none detectable in the protein hydrolysate. This sample also had the highest percentage of the infused activity observed in maternal plasma. Fetal plasma water has very little of the infused activity present, from 5 hours to 3 days having between 0.024 or 0.028 per cent present as phenylalanine and tyrosine. Total radioactivity increases slowly to between 0.4 and 0.45 per cent.

Maximum activity in maternal plasma (8 hours) of the leucine infused animal represented 6.15 per cent of the total infused activity. Fetal plasma of this experiment accounted for 0.47 per cent of the infused activity at its highest concentration (day 7). The maximum percentage of infused activity in maternal plasma water was 0.28 per cent and in fetal plasma water 0.06 per cent.

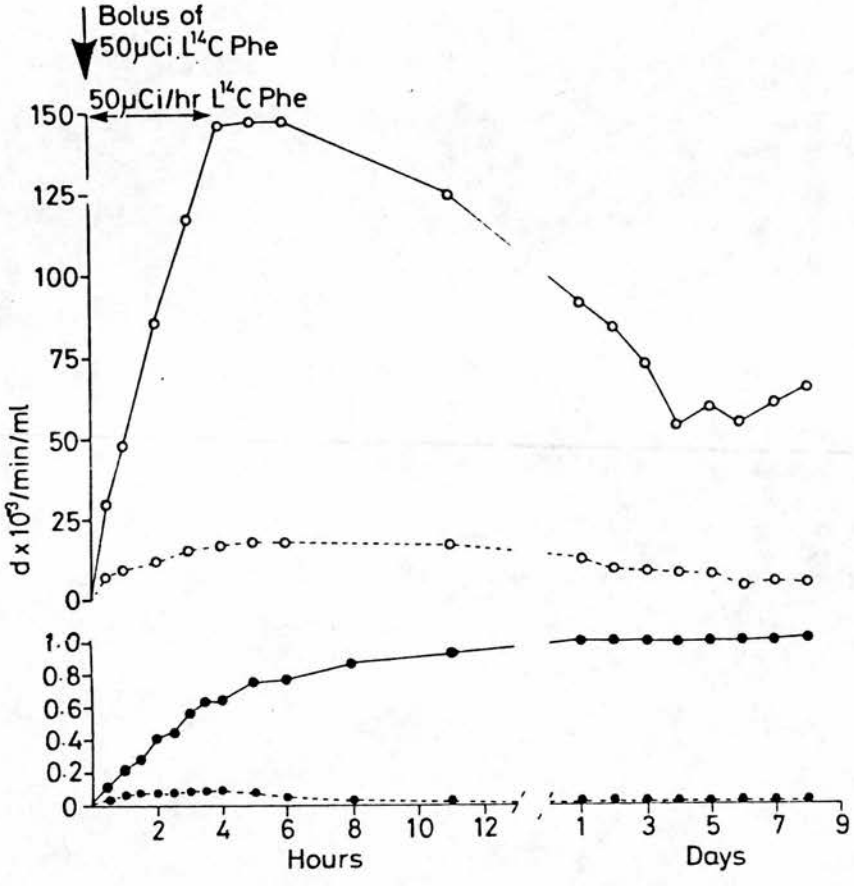


Fig 7i

Maternal (●) and fetal (○) activity in total plasma (–) and deproteinised plasma (---) during and after 250 μ Ci $L^{14}C$ phenylalanine infusion into the umbilical vein.

ii Fetal ^{14}C Infusions

Experimental Details

Experiment C3

Fetal Infusion LU ^{14}C Phenylalanine

LU - ^{14}C Phenylalanine 250 μ Ci in 25 ml isotonic saline was infused by syringe pump at 5 mls (50 μ Ci/hour) into the umbilical venous catheter for four hours after a bolus injection of 5 ml had been given at 11.30 hours. Maternal samples were taken from the jugular vein and fetal blood samples from the umbilical arterial catheter. The intervals of time between samples were as in experiment C1, but the experiment was terminated one day earlier. Fetal urine was obtained.

Results

a. Haematocrit values were within the range 26 - 30.5 per cent in maternal samples and 24 - 28 per cent in fetal blood samples.

b. Total and Deproteinised Plasma Ratio Activity Measurements

Transfer of labelled material from fetal to maternal circulation did occur (Fig 7i) but at a very slow and gradual rate, (0.16 x 10^3d/min/ml/hour). Deproteinised plasma radioactivity of maternal samples was very low, being only just above background after the infusion ended.

Fetal total plasma activity increased in a linear manner at 33.79 x 10^3d/min/ml/hour during the 4 hours of the infusion, but fetal deproteinised plasma increased only slowly after the 30 minute sample.

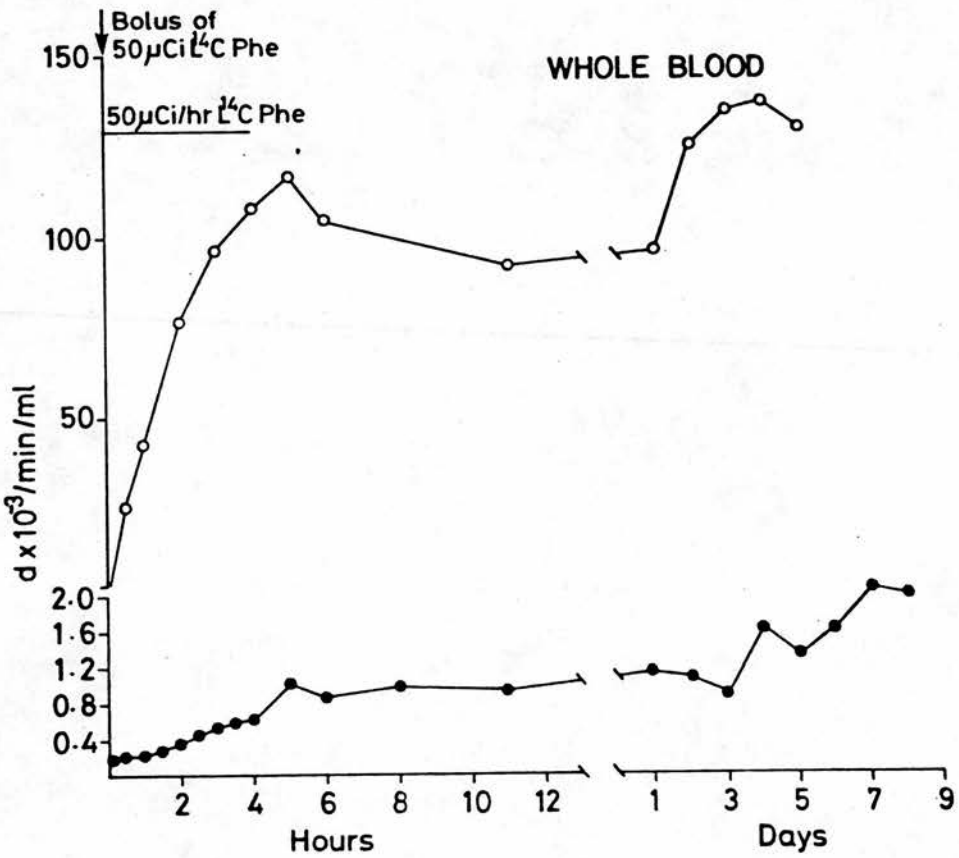


Fig 7ii

Maternal (●) and fetal (○) whole blood activity during and after 250 μ Ci L ^{14}C phenylalanine infusion into the umbilical vein.

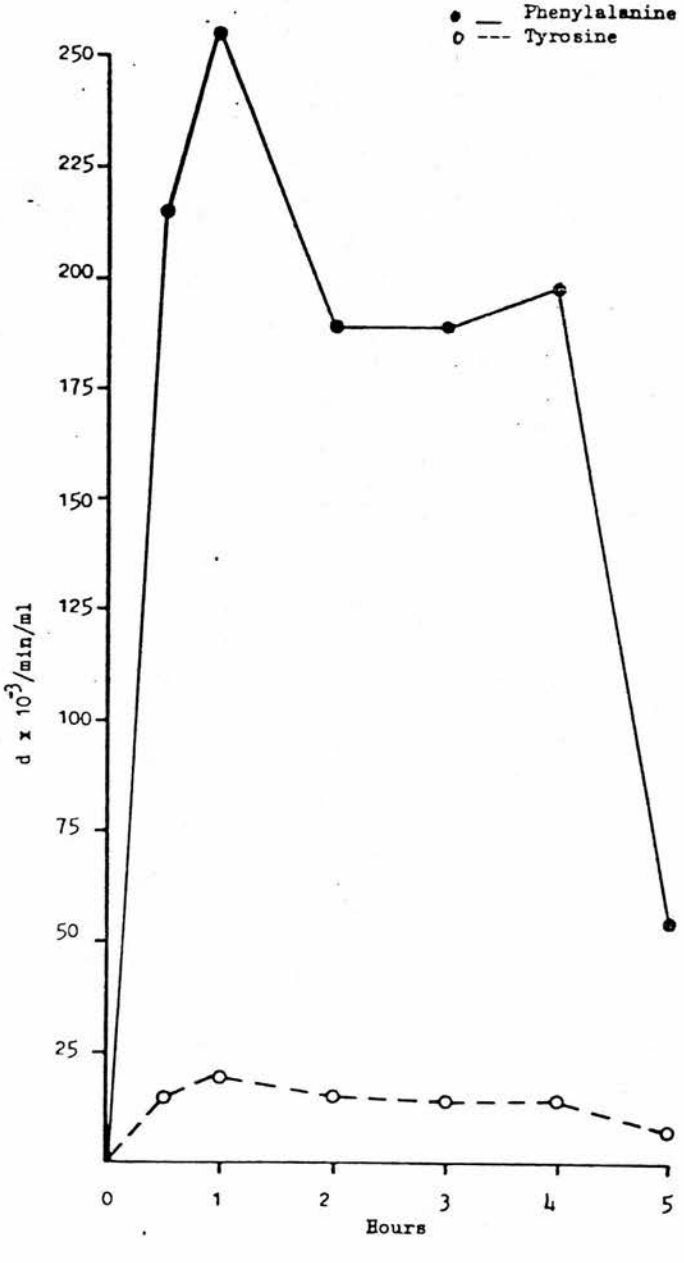


Fig 7iii

Fetal plasma water phenylalanine (-) and tyrosine (---) sA during and after 250 μ Ci L ^{14}C phenylalanine infusion into the umbilical vein.

c. Radioactivity in Whole Blood

The increase in whole blood radioactivity during the experiment is shown in Fig 7ii. Like maternal infusions the proportion of whole blood radioactivity associated with fetal RBC was low during the first 2 days (5 - 27 per cent) increasing to 71 per cent at experiment termination.

The proportion of activity associated with maternal RBC increased from approximately 25 per cent during infusion to 45 - 60 per cent in days 3 to 8.

d. Tissue Digestion, Allantoic Amniotic and Fetal Urine Radioactivity

Radioactivity associated with various fetal and maternal tissues is given on page 190.

Allantoic fluid recorded 6.06 x 10^3d/min/ml and amniotic fluid 1.23 x 10^3d/min/ml.

e. Specific Activity

The specific activity of free phenylalanine and tyrosine in fetal plasma water during the infusion and at one hour post infusion is shown in fig 7iii. The increase and decrease on starting and stopping the infusion were similar to the changes in maternal plasma SA during maternal infusions.

From the plateau SA fetal flux was calculated to be 100 µmol/Kg/hr.

f. Distribution of Radioactivity

Of the radioactivity infused into the fetus, samples taken between 2 and 6 hours showed 7.90 - 7.99 per cent of this activity to be in fetal plasma. A rapid rise to this value was observed (5.38 - 6.56 per cent at 30 minutes and 1 hour respectively) after which a slow decline from the plateau value followed (6.74, 4.98 and 3.97 per cent at 11 hours, 22 hours and 3 days respectively). Plasma water still accounted for 1.02 per cent of the infused activity at 11 hours and thereafter declined to 0.73 and 0.57 per cent at 22 hours and 3 days. Maternal plasma total activity exhibited a plateau activity of 0.25 - 0.32 per cent of the infused activity from hours 1 to 4 during infusion and thereafter increased very gradually to 0.66 per cent at 7 days. Maternal deproteinised plasma contributed less and less of the percentage activities with time, from 0.086 per cent at 30 minutes to 0.042 per cent at 4 hours and to 0.014 - 0.009 per cent between 11 hours and 7 days.

iii Dual ^{3}H and ^{14}C Infusion

Experiment C4

Simultaneous Infusion LU^{14}C phenylalanine and Fetal Infusion L^{3}H phenylalanine

Experimental Details

LU - ^{14}C Phenylalanine 175 μ Ci in 25 ml of mineral cocktail * (without dextrose) and 25 ml cocktail * (without dextrose) containing 250 μ Ci L^{3}H phenylalanine, each in separate 50 ml syringes placed in the same syringe pump, were infused into the maternal jugular vein and the umbilical vein respectively. A bolus of 5 ml of each solution was given simultaneously by manually pushing the syringe pump and infusion of both continued at 5 ml/hour for four hours (11.17 - 15.22 hours). therafter. Blood samples were taken after the same time intervals as in experiment C2 and the experiment continued for 8 days. Fetal urine was obtained.

* for composition see appendix 12

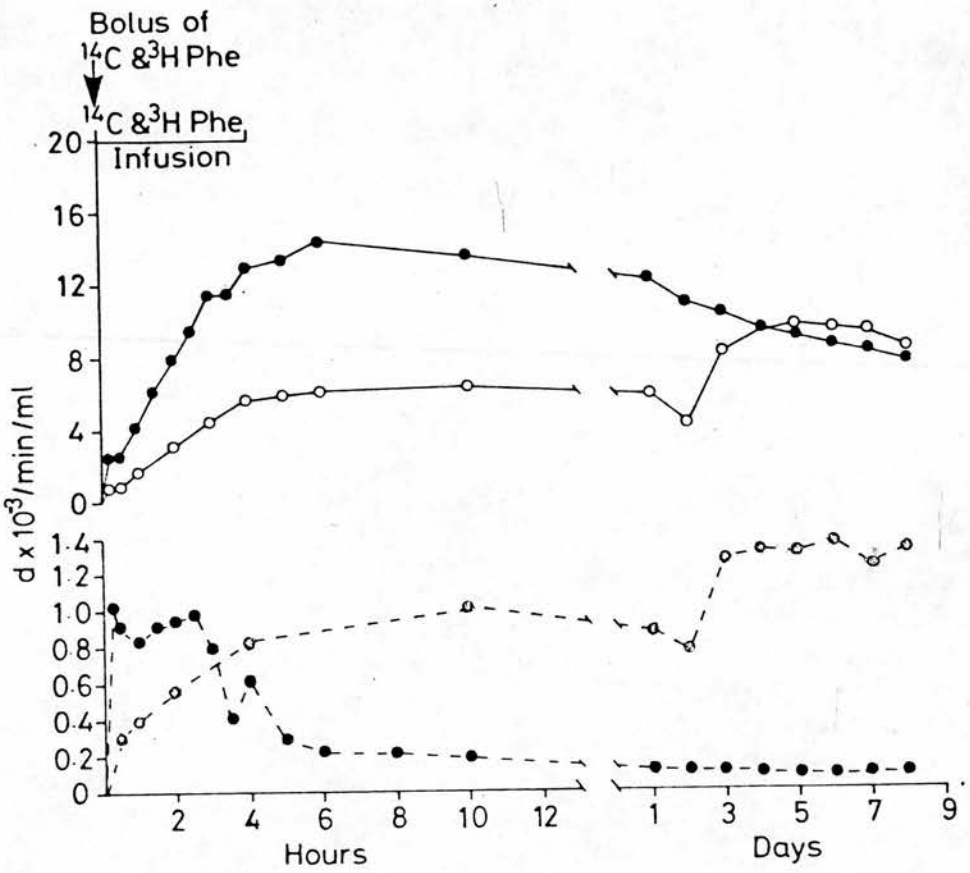


Fig 8i

Maternal (●) and fetal (○) plasma total (-) and deproteinised (---) ^{14}C activities during and after simultaneous infusion of 175 μ Ci L ^{14}C phenylalanine into the maternal jugular vein and 250 μ Ci L ^{3}H 2 - 3 phenylalanine into the umbilical vein.

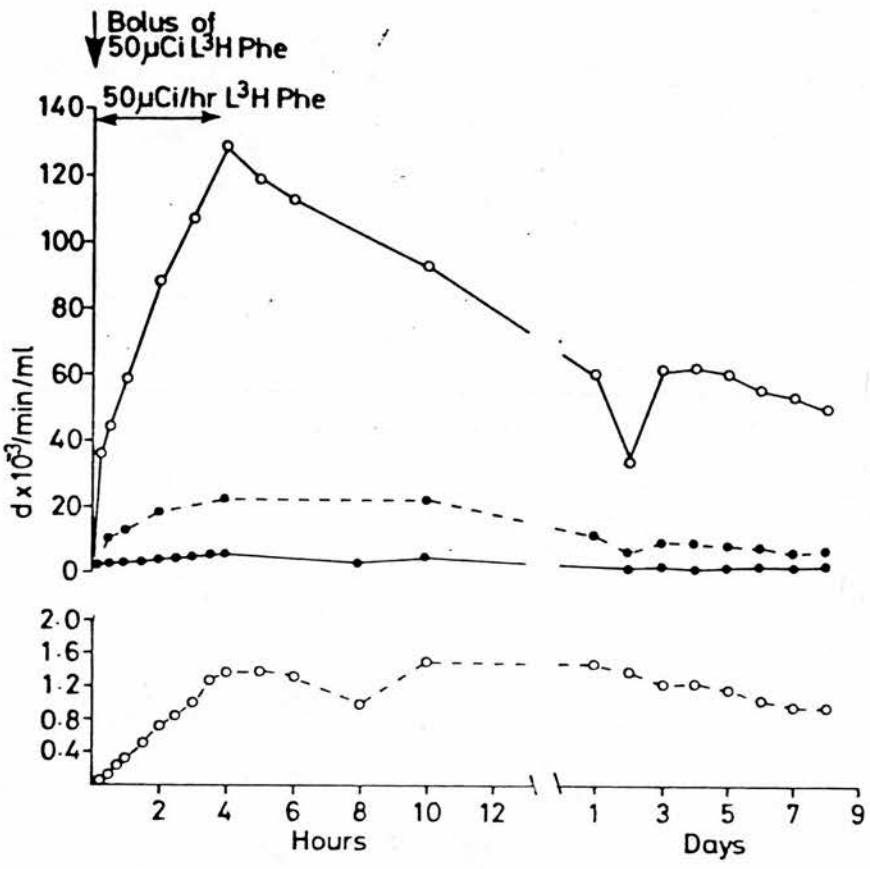


Fig 8ii

Maternal (●) and fetal (○) plasma total (–) and fetal (●) and maternal (○) deproteinised (---) ^{3}H activities during and after simultaneous infusion of 250 μ Ci $L^{3}H$ 2 - 3 phenylalanine into the umbilical vein and 175 μ Ci $L^{14}C$ phenylalanine into the maternal jugular vein.

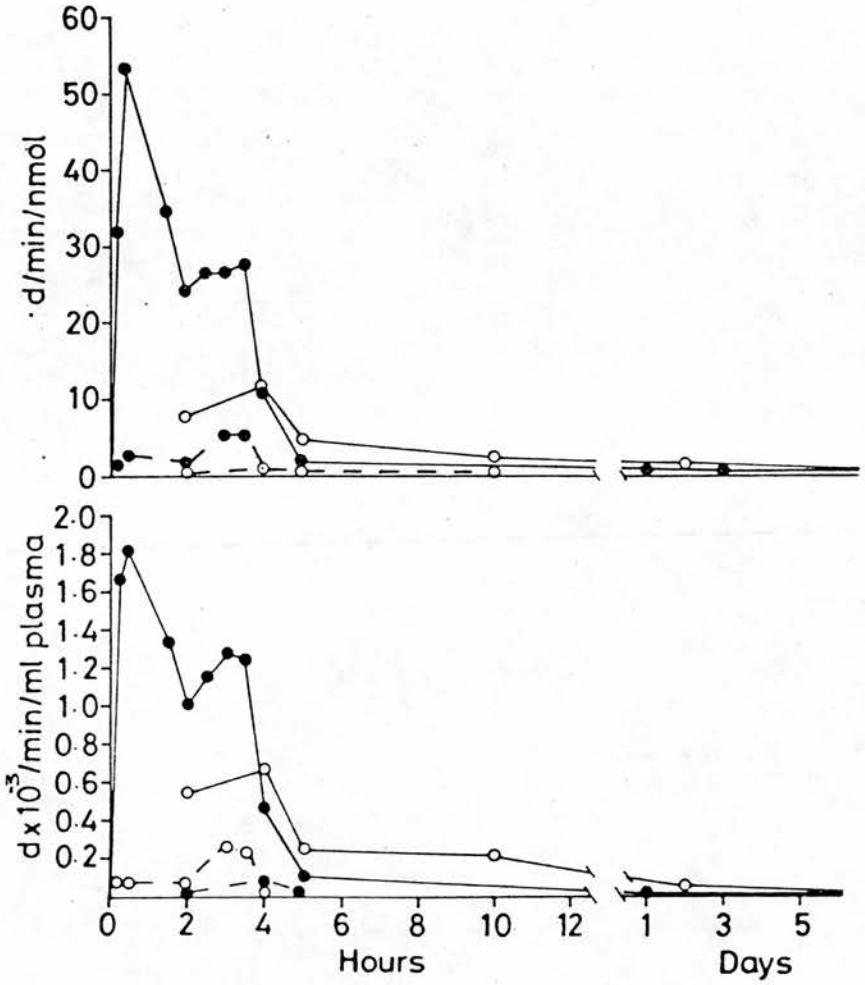


Fig 8iii

Maternal (●) and fetal (○) phenylalanine (–) and tyrosine (---) ^{14}C activity in plasma water (below) and free amino acid SA (above) during and after 175 μ Ci L ^{14}C phenylalanine and 250 μ Ci L ^{3}H 2 - 3 phenylalanine infusion into maternal jugular vein and umbilical vein respectively.

TABLE VI

Amino Acids Concentrations with Time

	Time (hr)	Tyrosine µmol/l	Phenylalanine µmol/l
Maternal	¼	46.0	51.7
	½	30.4	33.8
	1½	39.4	38.5
	2	42.2	41.5
	2½	40.2	43.5
	3	48.2	47.7
	3½	45.1	44.8
	4	40.1	42.7
	5	42.2	51.0
	22	47.5	36.9
	3 days	32.2	30.4
	7 days	30.0	39.2
Fetal	2	84.0	69.1
	4	66.6	58.7
	5	60.2	54.4
	10	85.5	96.5
	2 days	55.3	51.6
	7 days	52.4	57.3

Results

a. Haematocrit

Both maternal and fetal haematocrits were stable throughout the infusion, maternal values being between 27 and 30% and fetal values between 35 and 37% on the day of the infusion. On day 1 a fetal haematocrit of 73% was recorded, but thereafter fetal haematocrits were between 28.5 and 34% and maternal between 23 and 29%.

b. Total and Deproteinised Plasma Radioactivity Measurements

Total and deproteinised plasma radioactivity in maternal and fetal samples after maternal ^{14}C isotope infusion is shown in fig 8i and after fetal ^{3}H isotope infusion in 8ii.

c. Specific Activity Measurements

Plasma Water Values

Deproteinised plasma phenylalanine and tyrosine SA and the activity of these amino acids in one millilitre of plasma are shown in Figs 8iii (^{14}C) and 8iv (^{3}H). In Table VI the concentration of free phenylalanine and tyrosine in plasma is given.

a. ^{14}C Isotope

Maternal Samples

Phenylalanine SA increased rapidly to a peak value of 53.79 d/min/nmol at 30 minutes. Between 2 and 3½ hours after the bolus injection phenylalanine SA was at a plateau (24.29 - 27.90 d/min/nmol). However, although labelled tyrosine was detected at 15 minutes the tyrosine SA increased for a longer period of time than that of phenylalanine, and only two samples

at 3 and 3½ hours were of similar activities, 5.39 and 5.32 d/min/nmol respectively. Both phenylalanine and tyrosine decreased rapidly in the interval between the 3½ and 4 hour samples, suggesting that the infusion was effectively finished before the 4 hour sample was taken. Activity was undetectable in the tyrosine fraction from 5 hours post bolus injection, but labelled phenylalanine decreased more gradually, activity being just detectable at 22 hours and 3 days. The tyrosine:phenylalanine SA ratio increased from 0.07 in the 2 hour sample to 0.20 in the 3 hour sample, and like SA, decreased rapidly (from 0.19 to 0.05) in the time interval between the 3½ and 4 hour samples.

Fetal Samples

After 4 hours infusion the final phenylalanine SA(11.41 d/min/nmol) was greater than that of the maternal 4 hour sample (10.89 d/min/nmol) although the maternal SA had fallen rapidly from 27.90 d/min/nmol at 3½ hours. Unlike maternal phenylalanine SA, fetal SA increased appreciably between the 2 and 4 hour samples (7.62 to 11.41 d/min/nmol) after which there was only a slow decline to 4.60 d/min/nmol at 5 hours, 2.28 d/min/nmol at 10 hours and 0.35 d/min/nmol at 7 days. Tyrosine SA was also highest at 4 hours but both the increase to and decrease from the level (1.20 d/min/nmol) was slower, this being reflected in the tyrosine:phenylalanine SA ratio which reached a maximum at 10 hours.

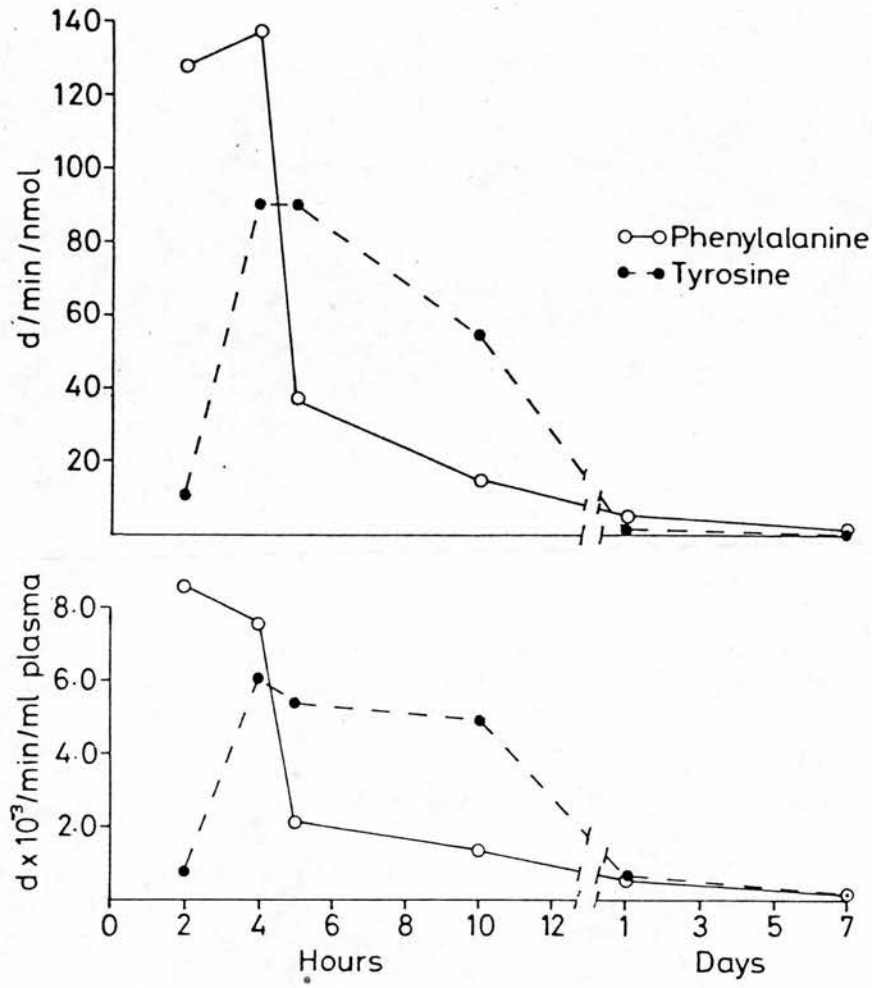


Fig 8iv

Fetal phenylalanine (-) and tyrosine (---) ^{3}H activity in plasma water (below) and free amino acid SA (above) during and after simultaneous infusion of 250 μ Ci L ^{3}H 2 - 3 phenylalanine into umbilical vein and 175 μ Ci L ^{14}C phenylalanine into the maternal jugular vein.

b. ^{3}H Isotope

Maternal Samples

Slow back diffusion of ^{3}H labelled phenylalanine was observed. The highest SA was 1.04 d/min/nmol in the sample taken 1½ hours after the bolus injection. ^{3}H activity was not detected in the tyrosine fractions.

Fetal Samples

Unlike the ^{14}C isotope, ^{3}H phenylalanine SA was at a plateau (123.9 - 128.8 d/min/nmol) between 2 and 4 hours and although, like ^{14}C SA, decreased between 4 and 5 hours, the decrease was faster. In the 5 hour sample ^{3}H SA was 29.4 per cent of the 4 hour value compared with 40.3 per cent for the ^{14}C isotope. The decrease in SA continued beyond 5 hours but activity was still detectable in the seventh day sample. No plateau SA was found in the tyrosine fractions, the SA increasing from 10.23 to 90.54 d/min/nmol between 2 and 4 hours. Unlike both the ^{14}C and ^{3}H phenylalanine SA, ^{3}H tyrosine SA was unchanged at 5 hours and in the 10 hour sample was still 63.9 per cent of the 4 hour sample and ^{3}H tyrosine was still detectable after 7 days. The highest tyrosine:phenylalanine SA ratio was present at 10 hours. Unlike the ^{14}C isotope appreciable activity was eluted from the ion exchange columns coincident with the elution of DOPA. Activity was 1.65, 1.94, 0.25 and 0.73 X 10^{3}d/min/ml plasma in the 5 and 10 hours, 2 and 7 day samples respectively. These fractions were not collected from the 2 and 4 hour samples.

iv Hydrolysates of Plasma Protein from Maternal L ^{14}C Phenylalanine (C1) and Simultaneous Maternal L ^{14}C and Fetal L ^{3}H Phenylalanine (C4)

Maternal ½, 2, 4, 22 hour and 3 and 7 days samples and fetal 5, 10 (C4) or 11 (C1) hour and 2 (C4) or 3 (C1) day samples were hydrolysed. In Experiment C4 maternal 15 minute and fetal 7 day samples were also hydrolysed.

Results

The ^{14}C radioactivity in the hydrolysed samples are shown in figs 9i and 9ii and the ^{3}H radioactivity in samples from the dual infusion in fig 9iii.

In both experiments maternal samples had detectable radioactivity in both the phenylalanine and tyrosine fractions after 2 hours, but only the experiment C4 sample had detectable radioactivity present at 30 minutes and this only in the phenylalanine fraction. Maximum radioactivity in the phenylalanine fraction was recorded at 22 hours but although this was also true of tyrosine in experiment C1, no increase at this time was found in experiment C4 (dual infusion).

In both experiments maximum fetal ^{14}C activity was found in the samples taken 6 - 7 hours after completing the infusion. In experiment C1 (maternal infusion) phenylalanine activity in protein increased between hours 4 and 11 from 150 x 10^{3}d/min/g to 227 X 10^{3}d/min/g protein although tyrosine activity appeared constant. Incorporation of unlabelled phenylalanine and tyrosine into protein would be expected to be increased or decreased to an equal extent. The divergence in SA could therefore be partially due to greater error at the lower activity level of tyrosine in samples but could also reflect decreased hydroxylation of phenylalanine to tyrosine - thereby lowering the precursor

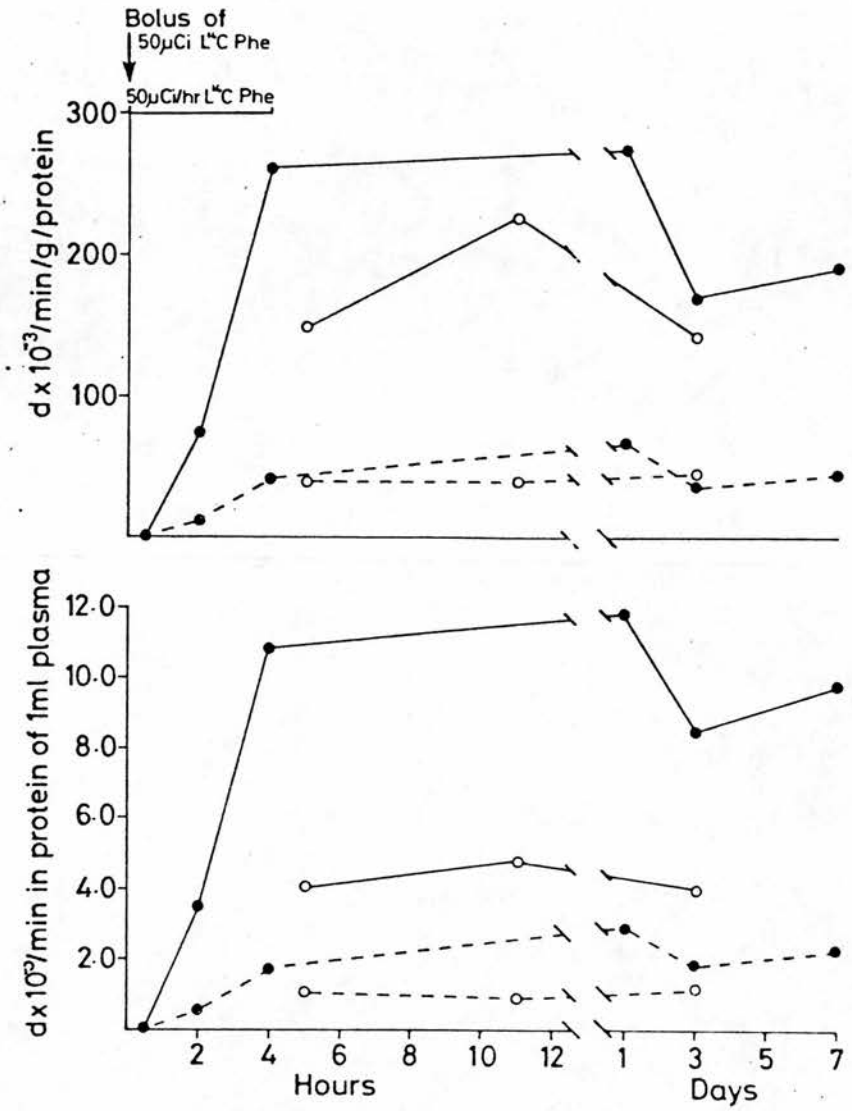


Fig 9.1

Maternal (●) and fetal (0) phenylalanine and tyrosine(---) activity in plasma protein from one millilitre of plasma (below) and per gram of protein (above) during and after 250 μ Ci $L^{14}C$ phenylalanine infusion into the maternal jugular vein

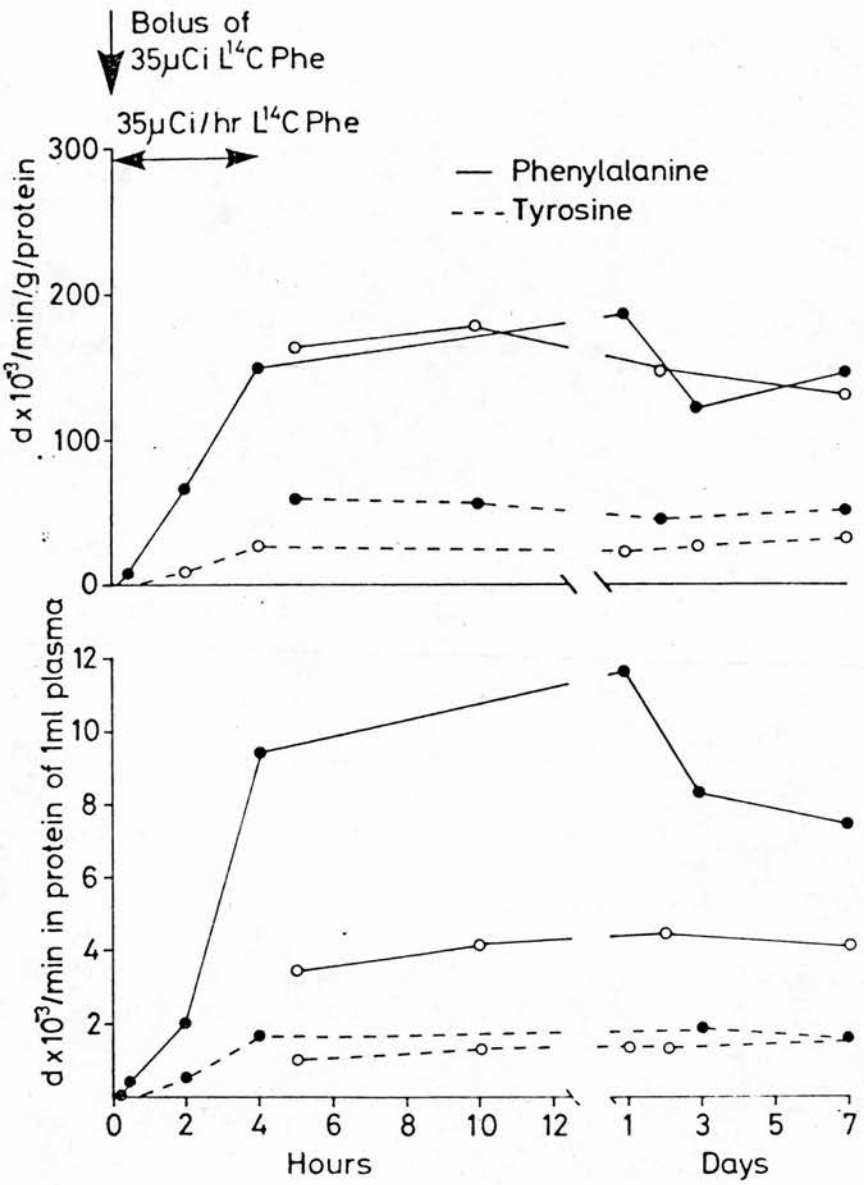
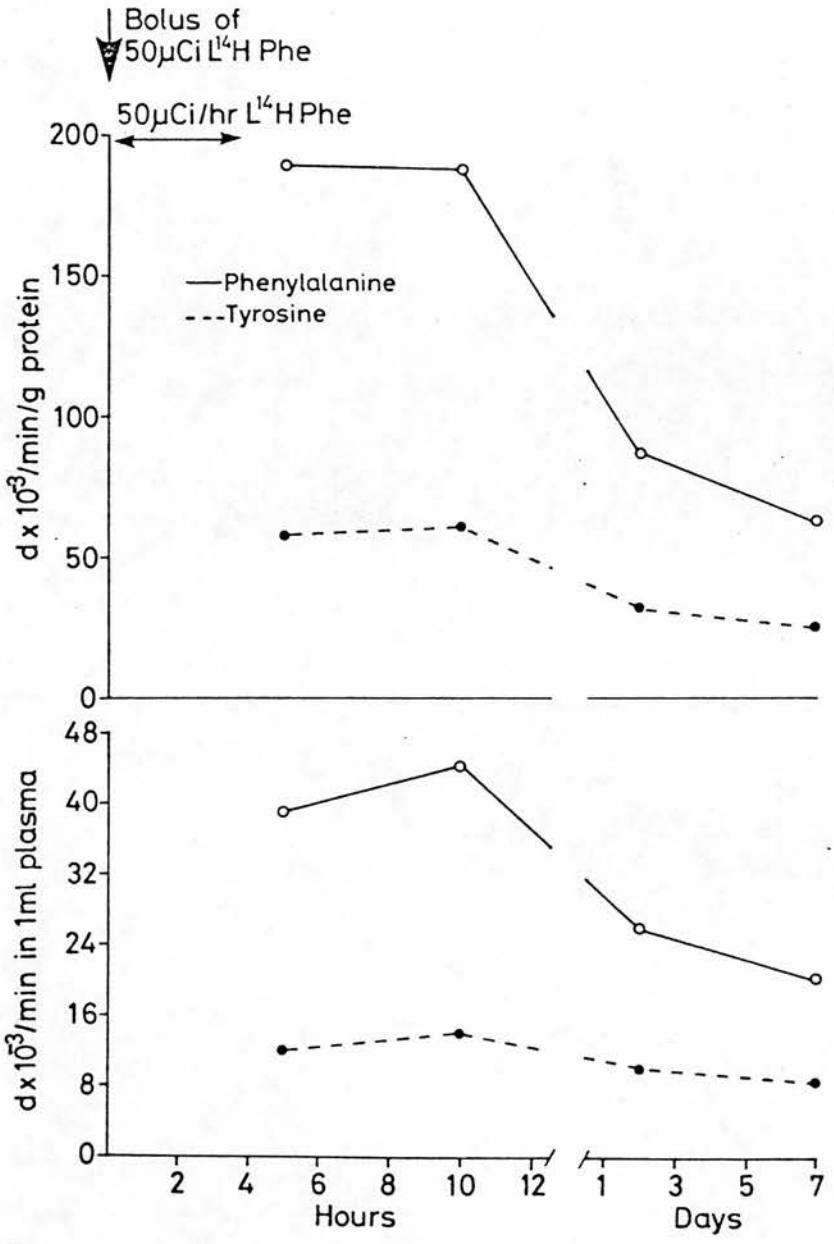


Fig 9ii

Maternal (●) and fetal (0) phenylalanine (-) and tyrosine (---) ^{14}C activity in plasma protein from one millilitre of plasma (below) and per gram of protein (above) during and after simultaneous infusion of 175 μ Ci L ^{14}C phenylalanine into maternal jugular vein and 250 μ Ci L ^{3}H 2 - 3 phenylalanine into the umbilical vein.



Fetal phenylalanine (–) and tyrosine (---) ^{3}H activities in plasma protein from one millilitre of plasma (below) and per gram of protein (above) after the simultaneous infusion of 250 μ Ci $L^{3}H$ 2 - 3 phenylalanine into the umbilical vein and 175 μ Ci $L^{14}C$ phenylalanine into the maternal jugular vein.

SA. Unlike maternal plasma in which the ratio of tyrosine SA: phenylalanine SA increased from hours 4 to 22, the small change observed in the fetal plasma was in the opposite direction - suggesting decreased hydroxylation of the ^{14}C phenylalanine. This was not true of Experiment C4, however, in which both maternal and fetal tyrosine:phenylalanine SA rations increased.

Tritium activity in the maternal phenylalanine and tyrosine fractions eluted from the ion exchange column was only a few counts above background and therefore too low for calculation of specific activities. Although the level of radioactivity per gram of protein was unchanged at 10 hours in the fetus there was a significant decrease by day 2. This contrasts with the ^{14}C hydrolysate measurements in which activity decreased only slowly. However, the ratio of tyrosine:phenylalanine activity per gram protein was similar to that of ^{14}C isotope, being 0.37 and 0.42 at 2 and 7 days respectively compared with 0.31 and 0.38 for the ^{14}C isotope.

Tissue Digestion

Activity of tissues (C4) are given in [illegible] (Page 190).

Flux

Maternal flux (^{14}C isotope) was calculated at 1374 μmol/hr

Fetal flux (^{3}H isotope) was calculated at 231 μmol/kg/hr

Flux is discussed in Chapter 4.

Discussion

(1) L ^{14}C Phenylalanine and L ^{14}C Leucine Activity

a. Maternal Infusions

In ^{14}C maternal infusion experiments including both L phenylalanine experiments C1 and C4 (Fig 5 and 9) and L leucine - Experiment C3 (Fig 8) fetal plasma water activity was greater than that of maternal plasma water reflecting the higher plasma free amino acid concentration in the fetus. Unlike the previous tritium experiments, in L ^{14}C phenylalanine and L ^{14}C leucine infusion. total activity increased both maternal and fetal plasmas for approximately 2 hours beyond the end of infusion. The continued increase in total activity with a simultaneous rapid decline in free plasma water activity suggests either a slow equilibration between plasma, tissue free amino acids and incorporated amino acids, or continued release of the pre-labelled proteins/peptides into the plasma over a prolonged period. However, immediate release of some proteins/peptides must occur as total plasma activity is greater than that accounted for by activity in plasma water and protein/total activity and shows a linear increase from the first maternal and fetal samples obtained after commencing infusion. This is in agreement with Peters[178] experiments in which labelled albumin was released from liver cells 15 minutes after incubation in labelled medium. In fetal lambs approximately one third of the placentally enriched blood has been reported to enter the fetal circulation directly via the ductus venosus and two thirds via the left lobe of the liver [307, 308]. Rapid hepatic uptake of the labelled amino acids could

therefore both reduce this concentration of labelled amino acids reaching peripheral tissues and facilitate the labelling of hepatic proteins e.g. albumin which may be subsequently released. The extended incorporation of ^{14}C isotope into the protein precipitated fraction could also suggest a continued "secretion" of labelled amino acid from the placenta. Complex formation of these labelled amino acid at a cell membrane site prior to protein synthesis (Hendler[152] Adamson[76]) or uptake of labelled amino acids by tissue cells, thereby maintaining the intracellular pool "reserve" of precursor amino acids could prolong the synthesis of labelled protein. Alternatively catabolism of prelabelled protein could similarly maintain the precursor pool radioactivity level. The predominant anabolic state of the fetus and the "drain" this causes on maternal reserves may favour the former mechanism in the fetus and the latter in the mother. Further evidence for the role of the placenta as a S.A reservoir was found in fetal infusions

b. Fetal Infusions

Infusing 250 μ Ci L phenylalanine directly into the fetus - experiment C2 - obviously resulted in a greater level of radioactivity in fetal plasma but the distribution of activity with time was similar to that observed in maternal experiments.

The abrupt end to the linear increase in total plasma activity contrasts with the continued increase in activity observed during maternal infusions. It supports the concept [26, 81, 82, 83, 84, 85, 115] that the placenta may accummulate labelled amino acids during infusion which are then secreted into the fetal circulation after the infusion had ceased [115].

Slow back diffusion, also observed in the tritium experiments, probably reflects slow exchange and passive diffusion down a concentration gradient from the fetus and placenta to the maternal circulation.

The continuous increase in deproteinised plasma activity contrasts with S A measurements (page255) the difference probably being explained by the presence of small peptides in the plasma water fraction. A continued release of these prelabelled unprecipitated peptides, which might also have a longer half life than amino acids could explain an increase in the plasma water activity from hours 4 to 5.

2 Specific Activity

The ^{14}C isotope of L phenylalanine was infused into the maternal jugular vein in experiment C1 and C4 In both experiments a rapid increase in maternal total plasma phenylalanine S A. occurred, but unlike activity measurements of tritium, plasma water equilibrium was not rapidly established - a suggestion of a plateau only being detected after 2 hours. This failure to rapidly establish a plateau level of S A is significant when planning radioactive infusion experiments as the plateau value of S A is an important measurement in calculating flux rates. Slow mixing of both the injected bolus and infused radioactivity with the plasma water free amino acid pool, or an unequilibrated system due to a steadily increasing rate of entry of amino acids into cells or fetal drain on maternal amino acids, could account for the slow attainment of plateau activity.

In both experiments, after stopping the infusion the S.A. of the free amino acid in plasma water decreased at a faster rate in maternal than fetal samples.

As the fetus is in an anabolic state it might have been thought that accelerated protein synthesis would deplete the free labelled amino acid pool faster than in the mother, but replenishment from the placental reservoir could maintain the fetal labelled free amino acid pool. Conversely in the mother uptake by the placenta could deplete the pool and subsequent proteolysis may further dilute the pool with unlabelled amino acids. Similarly incorporation of labelled amino acids into newly synthesized protein would (be expected to) diminish earlier in the fetus. However, this was not the case. The S A of hydrolysates, i.e. the ratio of labelled:cold phenylalanine, continued to increase in the plasma protein beyond the end of infusion, albeit at a slower rate. The abrupt halt in incorporation of fetal infused ^{3}H labelled amino acid into fetal protein in experiment C4 and in experiment C2 inferred from plasma total activity measurements would support the concept of continued replenishment of the protein precursor pool, as a slow increase in labelled protein would otherwise also have been expected in this experiment. The slight increase in maternal plasma protein S A would be unlikely to be due entirely to replenishment of labelled amino acids by back diffusion from the placenta. Although this could occur because fetal S.A. is greater than that of maternal plasma, the exchange equilibrium is towards the fetus. Utilisation of amino acids for "export" proteins and increased catabolism to meet fetal demands could contribute towards the maintenance of maternal plasma protein radioactivity levels.

Appearance of labelled tyrosine in the fetus could be a result of placental transfer of maternally prelabelled tyrosine alone or by direct hydroxylation of phenylalanine by the fetus or placenta. During maternal infusion the maternal plasma water S.A ratio of ^{14}C tyrosine: ^{14}C phenylalanine in experiment C4 varied between 0.046 and 0.063 and that of fetal plasma between 0.181 and 0.184. These are similar values to those found in experiment C1 - 0.008 - 0.040 and 0.10 - 0.149 respectively. This suggests that the fetus or placenta is capable of hydroxylating phenylalanine and contributes to fetal tyrosine. The tyrosine:phenylalanine ratios in experiment C4 are 0.12 - 0.23 for maternal protein (per g protein) and 0.31 - 0.38 for fetal protein, and in experiment C1 0.15 - 0.23 and 0.18 - 0.31 respectively, the higher ratios again being in the fetus. Efficient hydroxylation of phenylalanine to tyrosine within the placenta or fetal liver could explain the distribution of the tritium label when ^{3}H phenylalanine was directly infused into the fetus. In both maternal plasma water and plasma protein fractions of experiment C4 specific activity is too low to permit calculation of ratios but in the fetus plasma water tyrosine ^{3}H specific activity is surprisingly higher than ^{3}H phenylalanine specific activity from 4 hours to between 10 and 22 hours, the S.A. tyrosine:phenylalanine ratios in plasma water being 2.35 - 3.79. However, in plasma proteins the ratio 0.30 - 0.40 is very similar to the ^{14}C ratio (0.31 - 0.38). Continued hydroxylation of labelled phenylalanine (which was decreasing) to tyrosine, and or delayed release of labelled tyrosine from cells into plasma could explain the relatively slow decline of tyrosine compared with phenylalanine S.A.

Although ^{14}C tyrosine was present in fetal plasma water from the first fetal sample (15 minutes) of both the fetuses infused with L^{14}C phenylalanine (experiment C2 and experiment Ins C1 and therefore hepatic hydroxylation is likely, the ratio of ^{14}C SA tyrosine:phenylalanine (0.065 - 0.087 and 0.061 - 0.063 respectively) was closer to the maternal SA of the maternal infusions. This suggests that in the fetuses of the maternal infusions the increased ratio (greater than the sum of the maternal SA and the fetal SA in fetal infusions) resulted from both maternal and fetal hydroxylation and probably also hydroxylation in the placenta.

The high ^{3}H tyrosine SA in experiment C4 is therefore probably due to lability of the label rather than extensive fetal hepatic or placental hydroxylation, additional label being added to the phenylalanine molecule during its hydroxylation to tyrosine.

In man, infants born to phenylketonuric mothers have high phenylalanine but normal tyrosine plasma water concentrations suggesting that the enzyme phenylalanine hydroxylase is either inactive or unable to hydroxylate phenylalanine at a rate sufficient to maintain normal phenylalanine concentrations within the fetus. However back diffusion of tyrosine from fetus to mother (who would have low tyrosine concentrations) and continuous transfer of large quantities of phenylalanine to the fetus as a result of the high maternal phenylalanine concentrations could obscure any evidence of fetal hydroxylation determined by phenylalanine:tyrosine concentration ratios.

Conclusions

^{14}C Infusion

1. At equilibrium fetal plasma activity was greater than maternal plasma activity.
2. ^{14}C total plasma activity increases linearly in both maternal and fetal plasma.
3. Maternal infusion of L^{14}C phenylalanine and L^{14}C leucine resulted in both maternal and fetal total plasma activity increasing for 2 hours beyond the end of the infusion period.
4. There was an abrupt halt in the incorporation of activity into the acid precipitated plasma fraction after stopping L^{14}C infusions directly into the fetus.
5. The different patterns of total activity found after maternal and fetal infusions suggest that the placenta might act as a "reservoir".
6. Deproteinized fetal plasma activity increased throughout the fetal infusion of phenylalanine and continued for 1 hour beyond the end of infusion. Phenylalanine S A decreased rapidly between hours 4 and 5.
7. Maternal and fetal plasma water free amino acid S A reached a plateau level of activity approximately 2 hours after the bolus injection at the start of infusion.
8. Maternal plasma water S A decreased more rapidly than fetal plasma water S A at the end of infusion
9. "Back" diffusion of activity to the maternal circulation was slow.
10. The tyrosine:phenylalanine S A ratio is higher in fetal than maternal total plasma and plasma water.

(3) Comparison of Infusions using ^{3}H and ^{14}C Isotopes

The lability of the radioactive isotopes - ^{3}H and ^{14}C used to label amino acids has been found to differ[150,309]. The greater lability of tritiated amino acids compared with ^{14}C isotopes in these experiments may be confirmed by comparing the two series of results. In experiments T4 and C1 250 µ Ci L phenylalanine were infused into the maternal jugular vein. Total activity in maternal plasma reached 18.00 X 10^{3}d/min/ml using the ^{14}C isotope but only reached 13.60 X 10^{3}d/min/ml when tritiated phenylalanine was used. Maximum maternal total ^{3}H radioactivity was attained much faster (4 hours) than the ^{14}C peak total activity (6 hours). In addition, the distribution of label between bound and free forms was different - the ^{14}C peak plasma water activity being 1.40 X 10^{3}d/min/ml whilst tritium was almost double this at 2.40 X 10^{3}d/min/ml - i.e. 7 per cent of ^{14}C but 18 per cent of the ^{3}H label was present in the plasma water fraction. Plateau activity of plasma water from the tritiated infusion was also higher that that from the ^{14}C infusion - 2.20 X 10^{3}d/min/ml compared with 0.90 X 10^{3}d/min/ml - and the decrease from the plateau level was less - at 7 hours ^{3}H activity being 1.18 X 10^{3}d/min/ml (53 per cent of the ^{3}H plateau level) compared with 0.22 X 10^{3}d/min/ml (24 per cent of the ^{14}C plateau). This suggests that in the tritium experiment a greater proportion of the activity is present in water soluble freely labile compounds, possibly even in water

itself. The higher ^{3}H background count of the column effluent from S.A measurements of samples taken during the first 5 hours of experiment C4 - 24 d/min compared with that from samples taken at 10 hours (12 d/min) and 3 days (8 d/min) also suggests a high degree of label lability. As observed by Hider 1969[150] the tritium label appears to be lost during amino acid incorporation into protein. Assuming protein incorporation rates to be similar in the ^{14}C and tritium infusion, if the higher tritium plateau of plasma water reflected a higher S.A rather than label diffusion, an increased proportion of the precursor pool should be labelled and therefore higher total activities would be expected - the opposite to that which was found.

Comparison of ^{3}H and ^{14}C fetal infusions point to a similar interpretation. Although 500 μ Ci ^{3}H phenylalanine were infused compared with 250 μ Ci ^{14}C phenylalanine, by day 2 (the first day of ^{3}H fetal sampling) the fetal total plasma activity was 116.00 X 10^{3}d/min/ml compared with 80.00 X 10^{3}d/min/ml of the ^{14}C sample. In contrast, maternal total activity levels in the ewe infused with the tritium isotope reached 4.93 X 10^{3}d/min/ml at 11 hours compared with 0.90 X 10^{3}d/min/ml in the comparable ^{14}C samples. At 48 hours the respective values were 5.53 X 10^{3}d/min/ml and 1.00 X 10^{3}d/min/ml.

One could speculate that a higher plasma water plateau activity in fetal plasma during and continuing beyond the end of infusion of tritiated phenylalanine increased the diffusion of label across the placenta to the maternal circulation and thereby increased the maternal plasma activity.

Conclusions

Comparison of ^{3}H and ^{14}C Phenylalanine Infusion

1. L 2 - 3 ^{3}H phenylalanine infusion resulted in higher plasma water and lower total plasma activities than L ^{14}C phenylalanine infusion.
2. Maximum maternal total plasma ^{3}H activity was obtained faster than ^{14}C activity.
3. Distribution of activity between plasma total (representing the amino acid incorporated into protein) and plasma free amino acid activity differed with ^{3}H and ^{14}C infusions - 7 per cent of the ^{14}C but 18 per cent of the ^{3}H activity being present in plasma water.
4. In the 7 days after the infusion, plasma water activity remained at a higher level of activity in the ^{3}H infusion than in the ^{14}C infusion.
5. Infusion of L 2 - 3 ^{3}H phenylalanine into the fetus resulted in higher maternal total radioactivity than when L ^{14}C - U-phenylalanine was similarly infused.

TABLE VII

Physical Details of Sheep and Route of Infusion Employed

Experiment	Sheep No,	No. of fetuses	Condition	Weight kg.	Gest. days	Days post operations	Route of Infusion	Amino Acid
Ins C1	K 944	1	live	2.740	124	14	mv	^{14}C Lys
Ins C2	K 651	2	live live	2.160 2.110	125	11	uv	^{14}C Phe
Ins C3	K 659	1	live	3.650	126	16	uv	^{14}C Leu
Ins C4	H 137	1	live	2.910	125	15	uv	^{14}C Lys
*Ins C5	K 947	1	live	2.940	126	15	*amniotic fluid	^{14}C Lys

* Although it was originally thought that the infusion had been into the umbilical artery, on sacrifice, it was found to have been into the amniotic fluid.

Phe = L phenylalanine

Leu = L leucine

Lys = L lysine

Gest = Gestation

Part IV

$L^{14}C$ Amino Acid Infusions with Insulin

In these experiments each fetus acted as its own control, $L^{14}C$ amino acids being infused for a four hour period into the fetus (3 experiments) or mother (1 experiment) prior to infusing insulin into the fetus. Because of the delay in establishing plateau S A in the primed experiments of the previous year, the bolus quantity and infusion rate were reduced, from 50 μCi and 50 μCi/hr to 30 μCi and 30 μCi/hr. Infusion of the labelled material directly into the fetus increased the S A of the free amino acid in plasma and reduced the effect of placental metabolism as opposed to transfer of the amino acid. Three fetal and one maternal infusion were carried out. Additionally one infusion into the amniotic fluid is reported.

$LU^{14}C$ phenylalanine and $LU^{14}C$ leucine were identical with those used in year 2. $LU^{14}C$ lysine monohydrochloride had a specific activity of 330 m Ci/mmol and a radioactive concentration of 50 μ Ci/ml. It was obtained from the Radiochemical Centre, Amersham. Crystaline insulin 20 iu/ml (Burroughs Wellcome and Co., London) was diluted to give a final concentration of (2 iu/12.6 ml) in isotonic saline containing 1 per cent low salt human albumin (Scottish Blood Transfusion Service). The pump was calibrated to deliver 4.2 ml/hour from a 50 ml syringe, the pump holding 2 syringes. During the first four hours amino acid plus saline solutions were infused. At four hours the saline syringe was replaced with the one containing insulin, excess solution being expelled as the syringe was fitted into the pump. After

a further three hours infusion with amino acid and insulin solutions, (0.66 iu insulin/hour) the insulin syringe was replaced by a syringe containing saline and infusion continued until the syringes were emptied approximately 3 - 4 hours later. Animals were then immediately sacrificed. Amniotic and allantoic fluids were aspirated into syringes. Tissues from the first four experiments were taken for autoradiography and from the fifth for tissue counting and autoradiography. Tissues taken for autoradiography were:- adrenal, kidney, liver, spleen, muscle, heart, thyroid and for tissue counting fetal and maternal liver, spleen and kidney. Placental cotyledons were taken for both tissue counting and autoradiography from all experiments. Two cotyledons from each experiment were separated into maternal and fetal components before storing at -70°C. Fetal urine was obtained from experiments Ins C2,4. Table VII gives details of the animals, amino acids and routes of infusion used in these experiments.

i Maternal Amino Acid and Fetal Insulin Infusion

Experimental Detail

Experiment Ins C1

Maternal Infusion LU ^{14}C Lysine and Fetal Infusion of Insulin

LU - ^{14}C Lysine 550 μ Ci in 46.2 ml was infused into the maternal jugular vein and saline, with or without insulin was infused into the umbilical vein. As before, infusion and bolus injections were at 4.2 ml/hour and 4.2 ml respectively. Infusion was started at 00.10 hours and the saline/insulin at 04.17 hours. Insulin was stopped at 07.27 hours and the infusion finished at 09.41 hours. Maternal and fetal blood samples were taken at $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3, 4 (preinsulin) $4\frac{1}{4}$, $4\frac{1}{2}$, 5, 6, 7 (insulin) and $7\frac{1}{4}$, $7\frac{1}{2}$, 8 and 9 hours (post insulin). Maternal blood samples were also taken at $1\frac{1}{2}$, $2\frac{1}{2}$, $3\frac{1}{2}$ hours and blood fluoride tubes collected at 2, 4, 6, 7, 8 and 9 hours.

Results

a. Haematocrit

Between hours 1 and 2 the maternal haematocrit fell from 40.5 (at zero time 42.5) to 35.5 per cent. Values from samples taken after the second hour were between 30.0 and 33.5 per cent. Fetal values declined slowly from 41.5 per cent at zero hours to 34 per cent at 10 hours.

b. Plasma Glucose

TABLE VIII

Time Hours	Plasma Glucose mmol/l		Insulin ng/ml	
	Maternal	Fetal	Maternal	Fetal
0	-	-	-	0.63
2	0.98	0.95	-	-
4	1.21	0.69	-	0.81
4½	-	-	-	5.94
6	2.58	0.56	-	10.46
7	0.04	0.56	1.37	11.42
7½	-	-	-	7.82
8	0.94	0.64	-	-
9	0.63	0.77	1.64	1.44

Fetal plasma glucose concentrations were lower during the period of insulin infusion, but the decrease was not as great as that of maternal plasma glucose which decreased markedly between hours 6 and 7. This may be explained by increased fetal demand for glucose during the period of insulin infusion, increasing placental transfer and thereby decreasing the maternal concentration. However, because of the relative size of fetal demand (small) to that of the maternal plasma glucose pool (large) this explanation would seem unlikely.

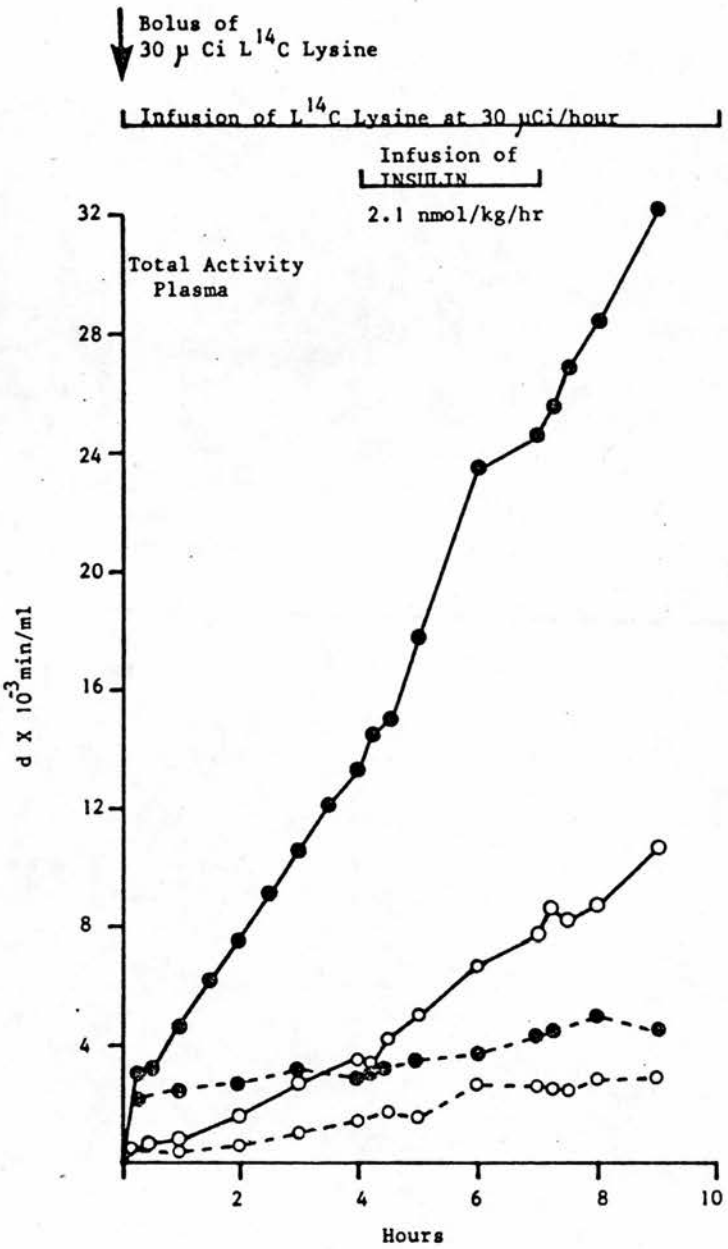


Fig 10i

Maternal (●) and fetal (O) total (-) and de-proteinised (---) activities during maternal continuous infusion of $L^{14}C$ lysine (30 μ Ci/hr) and fetal infusion of insulin between hours 4 and 7.

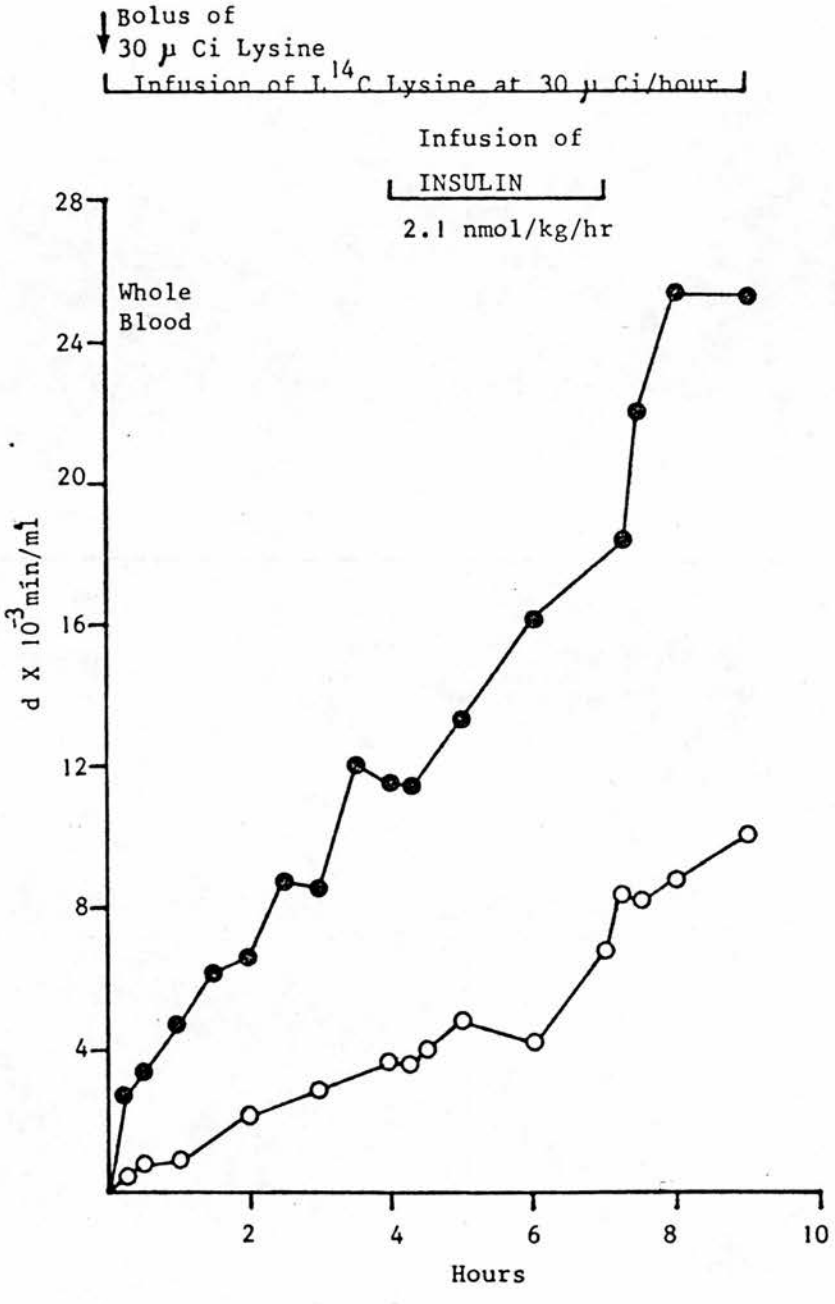


Fig 10ii

Maternal (●) and fetal (0) whole blood activities during continuous maternal infusion of $L^{14}C$ lysine (30 μ Ci/hr) and fetal infusion of insulin between hours 4 and 7.

c. Insulin Values

The greatest change in plasma insulin concentrations occurred within the first 30 minutes of infusion. Thereafter the concentration doubled. An immediate decrease on terminating insulin infusion was noted, but pre infusion concentrations were not regained even at the end of the experiment (9 hours).

d. Total and Deproteinised Plasma Radioactivity Measurements Fig 10i

There was an increase in the rate of accummulation of radioactivity in maternal plasma for 2 hours after the start of the insulin infusion, but after terminating the insulin infusion the rate of accummulation returned to that of the pre-insulin basal period. Deproteinised plasma radioactivity in maternal samples also increased slowly on infusing insulin.

Fetal total plasma activity, although at a lower level, changed in a manner similar to that of maternal samples for the first 7 hours, but did not return to the pre-insulin rate of accummulation. Radioactivity in deproteinised plasma increased between Hours 5 and 6 to a new plateau at which it remained until the experiment was terminated.

e. Radioactivity in Whole Blood Fig 10ii

No change was found in the rate of accummulation of radioactivity in maternal whole blood until insulin infusion stopped, when the rate increased for 2 hours. The rate of increase in radioactivity in fetal whole blood however was most unstable during the insulin infusion.

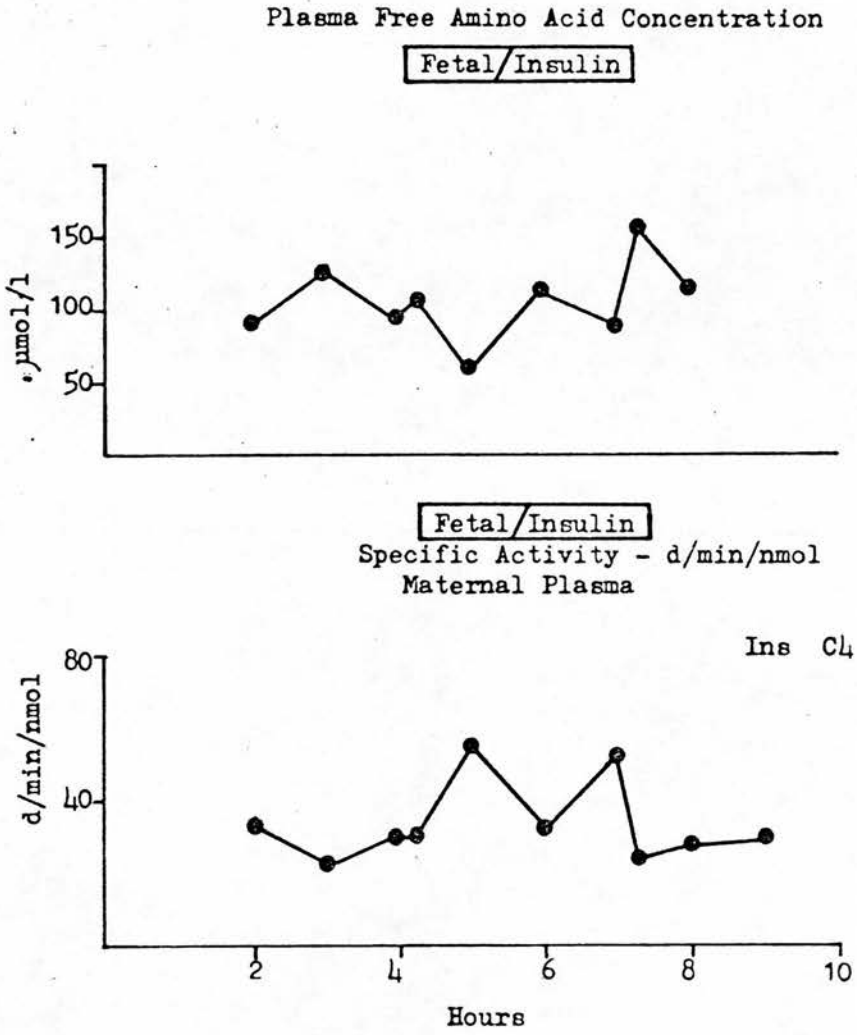


Fig 10ii

Maternal plasma water lysine SA (below) and concentration (above) during maternal continuous infusion of $L^{14}C$ lysine (30 µ Ci/hr) and fetal infusion of insulin between hours 4 and 7.

f. Specific Activity Measurement

Maternal lysine SA and concentration is shown in Fig 12ii. Between hours 2 and 4 plasma lysine SA fluctuated between 22.2 and 33.6 d/min/nmol, but from 15 minutes post insulin until the end of the insulin infusion, fluctuation was between 30.8 and 54.7 d/min/nmol, the highest SA and lowest plasma amino acid concentration being recorded 1 hour post insulin. Conversely lowest SA and the highest lysine concentration was recorded 15 minutes after stopping insulin.

g. Tissue Digestion

Radioactivity measurements of placental tissue are given in [illegible].

h. Flux

During the first 4 hours of lysine infusion maternal flux was calculated at 2220 μmol/hr (See Chapter 4).

ii Fetal Amino Acid and Insulin Infusion

Experimental details

Experiment Ins C2

Fetal Infusion LU ^{14}C Phenylalanine and Insulin

LU - ^{14}C Phenylalanine 330 μ Ci in 46.2 ml of isotonic saline was infused into the umbilical venous catheter at 4.2 ml/hour. In addition saline with or without insulin was infused from the same pump - giving a total infusion volume of 8.4 ml/hour. A bolus of 4.2 ml from each syringe was given by depressing the pump. Infusion commenced at 10.03 hours, insulin being given from 13.45 hours until 16.58 hours and the infusion stopped at 18.59 hours. Dextrostix measurement of blood glucose were made and maternal samples for biochemical analysis of blood glucose taken at 5,6,7,7½ and 9 hours and fetal samples at 5,6,7,7¼, 8,9 and 10 hours. Maternal and fetal blood samples were taken at ¼,1,2,3,4 (preinsulin) 4¼,5,6,7,7¼,8,9 and 10 hours (postinsulin). Maternal blood samples were also taken at ½, 1½,2½,3½,3½ and 7½ hours post bolus injection.

Experiment Ins C3

Fetal Infusion LU ^{14}C Leucine and Insulin

LU ^{14}C Leucine 330 μ Ci and insulin were infused into the umbilical venous catheter under conditions identical with those in experiment Ins C1. The leucine infusion commenced at 08.30 hours and insulin at 12.40 hours. Insulin stopped at 15.42 hours and the leucine infusion finished at 18.23 hours. Maternal blood samples were taken at the same time intervals as in experiment Ins C1 but additional fetal samples were taken at ½,4½ and 7½ hours. Fetal samples for blood glucose were taken at 4 and 7 hours.

Experiment Ins C4

Fetal Infusion LU ^{14}C Lysine and Insulin

LU - ^{14}C Lysine 330 μ Ci was infused into the umbilical venous catheter during 9½ hours, with insulin being given for 3 hours, 4 hours after commencing. Infusion was started at 08.16 hours after the initial bolus of 4.2 ml given at 08.12 - 08.16 hours. Saline and insulin replaced the saline from 12.29 until 15.31 hours and the infusion was terminated at 17.50 hours. Maternal and fetal blood samples were taken as in experiment Ins C2, 3, and 4, and blood fluoride tubes at 4, 7 and 9½ hours after the experiment commenced.

Results

a. Haematocrit

In Exp Ins C2 Maternal haematocrit fell from 31.0 per cent at the commencement of the experiment to 27.0 per cent in the final sample, fluctuations between these values occurring during the procedure. Fetal haematocrit exhibited a much greater fall from 48.5 to 32.5 per cent. However, as the 7¼ hour sample had a haematocrit of 45.5 per cent and values of samples previous to this, with the exception of one (6 hours - 38.5 per cent) were above 40.0 per cent it is probably only within the last 3 hours that adaptation to blood loss might significantly affect results.

/of Exp Ins C3

Maternal haematocrit values remained steady between 30 and 31 per cent apart from the 8 hour haematocrit (28 per cent) and 9 hour (29 per cent) samples. Fetal haematocrits also remained within a narrow range only three values falling outwith the range 38.5 - 40 per cent. These three were the 2 hour, 3 hour and 7¼ hour samples, which gave values of 42.5, 41 and 37.5 per cent respectively.

Haematocrits of Ins C4 samples remained reasonably constant.

The maternal haematocrit fell from 37 to 30 per cent between hours 1 and 2 and thereafter remained between 30 and 33 per cent. The fetal haematocrit remained very constant - between 34 and 36 per cent - after the initial and greatest fall from 37 to 35 per cent in the first 15 minutes.

TABLE IX

PLASMA GLUCOSE AND INSULIN CONCENTRATIONS

	Glucose mmol/l						Insulin ng/ml					
	Ins C2		Ins C3		Ins C4*		Ins C2		Ins C3		Ins C4	
Time (Hr)	M	F	M	F	M	F	M	F	M	F	M	F
0	-	-	-	-	-	-	-	0.57	-	1.03	-	1.23
4	-	-	-	0.69	1.38	0.39	-	6.12	-	2.71	-	1.76
4¼	-	-	-	-	-	-	-	7.36	-	-	-	-
4½	-	-	-	-	-	-	-	-	-	6.36	-	7.64
5	1.89	0.49	-	-	-	-	-	-	-	-	-	-
6	1.49	0.49	-	-	-	-	-	27.42	-	6.80	-	13.12
7	2.34	0.09	-	0.59	1.12	ND	0.65	32.20	1.00	8.68	2.60	17.47
7¼	1.44	0.15	-	-	-	-	-	4.53	-	-	-	-
7½	-	-	-	-	-	-	-	-	-	6.80	-	6.90
9	2.30	0.15	-	-	-	-	0.49	2.27	-	-	-	-
10	-	0.09	-	-	1.33	0.31	-	-	1.30	3.86	1.45	1.09

M = Maternal
F = Fetal

* ppt with 3% perchloric acid

ND - non detected

b. Plasma Glucose and Insulin Values

The concentrations of blood glucose and plasma insulin are given in Table IX. Fluctuations in maternal glucose concentrations were unconnected with the infusion of insulin into the fetus. The fetal plasma glucose concentrations decreased significantly during the insulin infusion in experiments Ins C2 and Ins C4, but was only slightly lower in the leucine infused fetus. Although the glucose concentrations recovered to the pre-insulin value in the lysine (Ins C4) infused fetus, in the fetus infused with phenylalanine plasma glucose was still low at the end of the experiment.

Fetal insulin concentrations increased rapidly during the insulin infusion, but the percentage increase above baseline concentrations varied. Likewise although insulin concentrations decreased rapidly on withdrawing insulin, the degree and rate of disappearance of insulin from the plasma varied, with baseline concentration only being regained in experiment Ins C4 (lysine infusion).

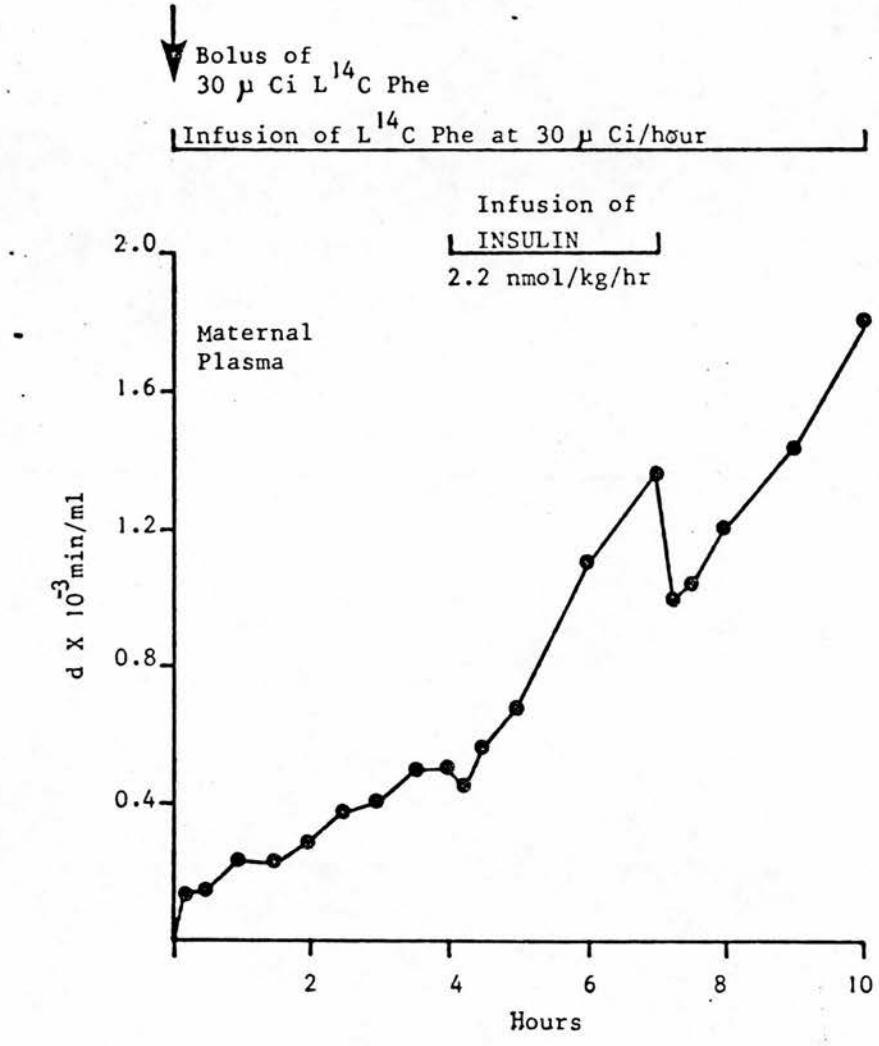


Fig II ia

Maternal plasma activity during continuous fetal infusion of $L^{14}C$ phenylalanine (30 μ Ci/hr) and insulin infusion between hours 4 and 7

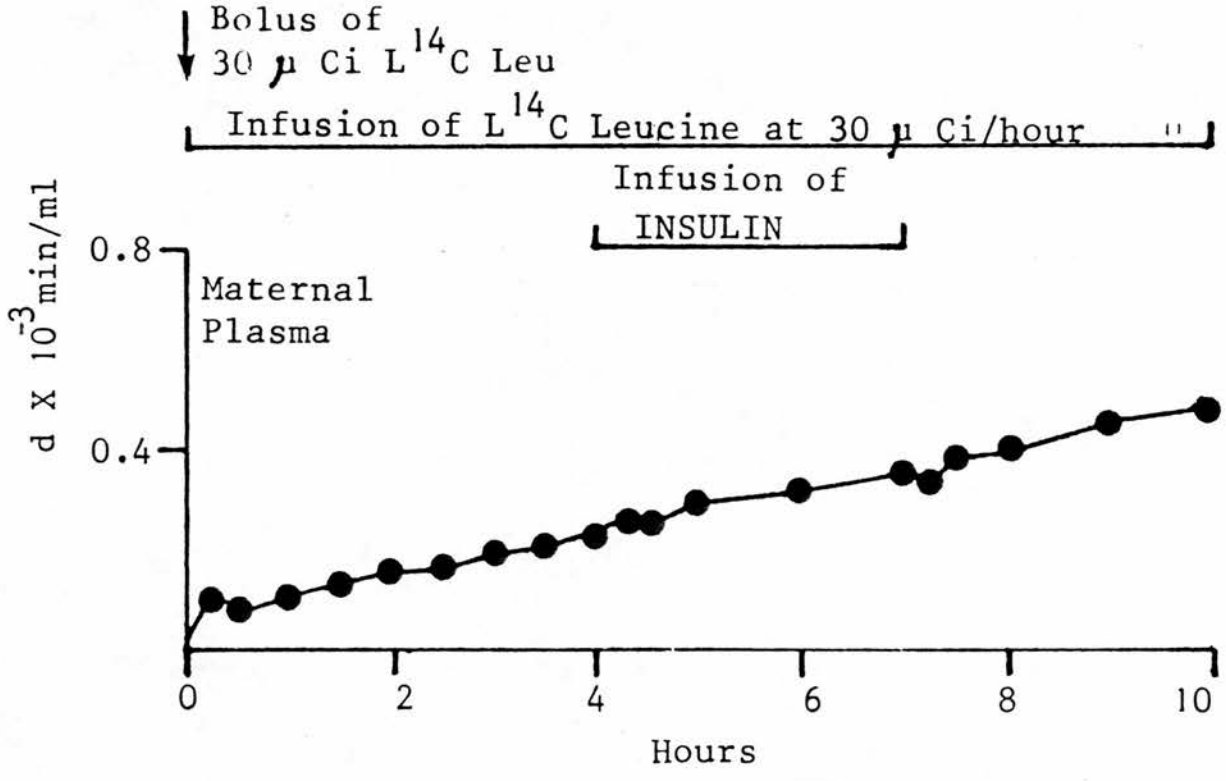


Fig 12ia

Maternal plasma activity during continuous fetal infusion of $L^{14}C$ leucine (30 μ Ci/hr) and insulin infusion between hours 4 and 7.

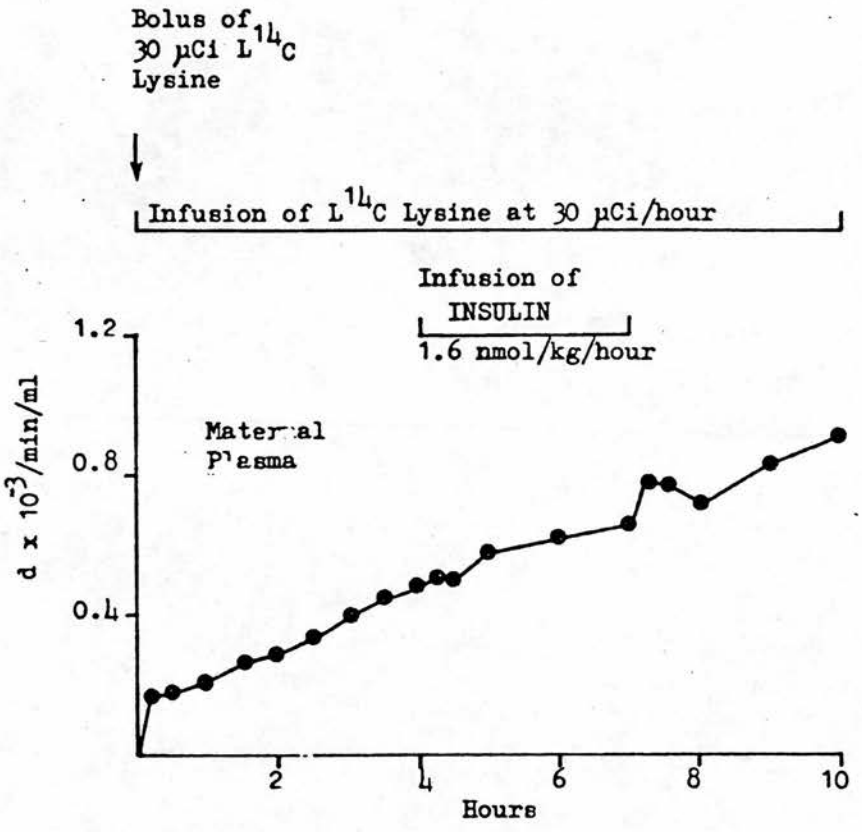


Fig 13 ia

Maternal plasma total activity during continuous fetal infusion of $L^{14}C$ lysine (30 µ Ci/hr) and infusion of insulin between hours 4 and 7.

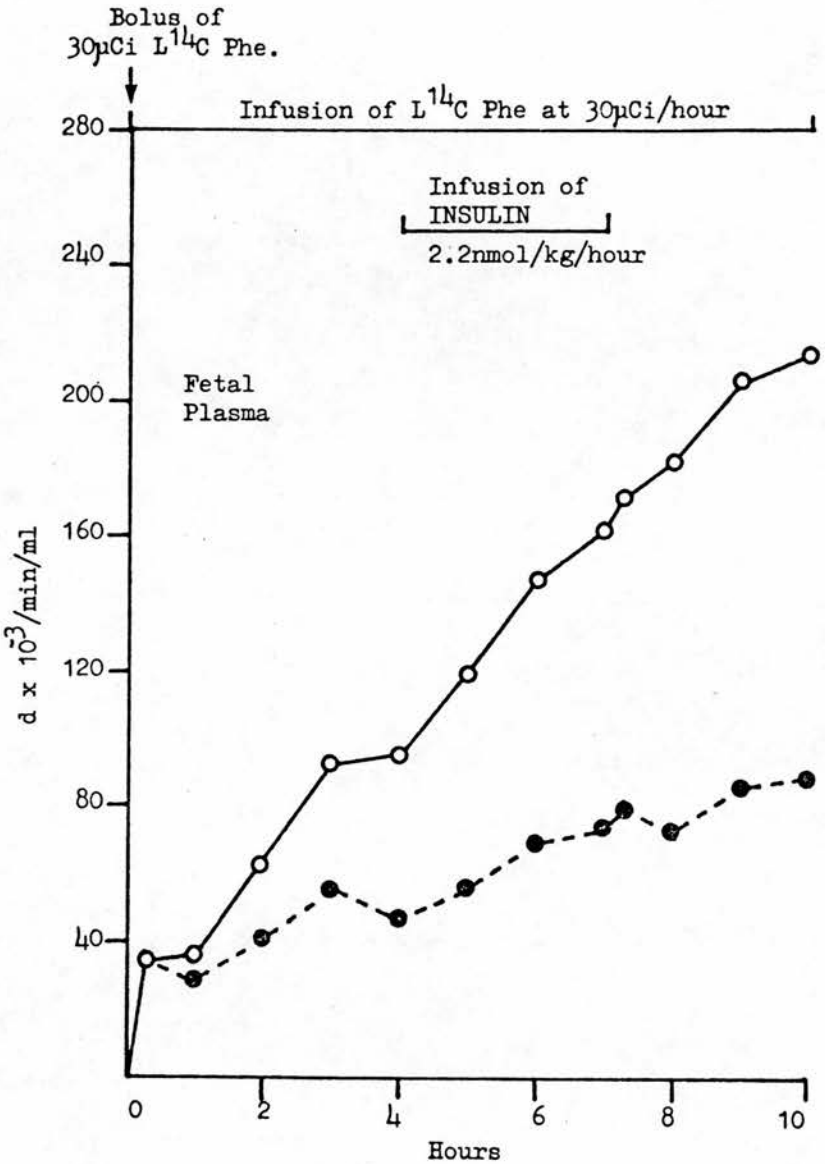


Fig IIib

Fetal plasma total (0) and deproteinised (●) activity during continuous fetal infusion of $L^{14}C$ phenylalanine (30 μ Ci/hr) and insulin infusion between hours 4 and 7.

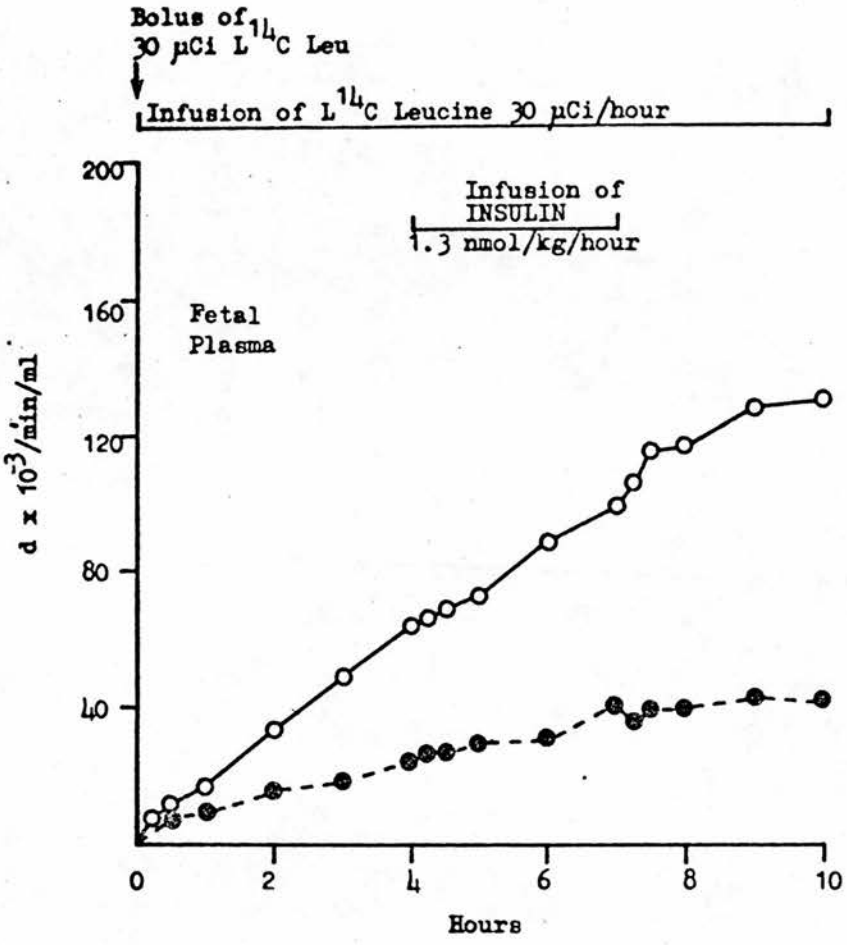


Fig 12ib

Fetal plasma total (0) and deproteinised (●) activity during continuous fetal L ^{14}C leucine infusion (30 µ Ci/hr) and insulin infusion between hours 4 and 7

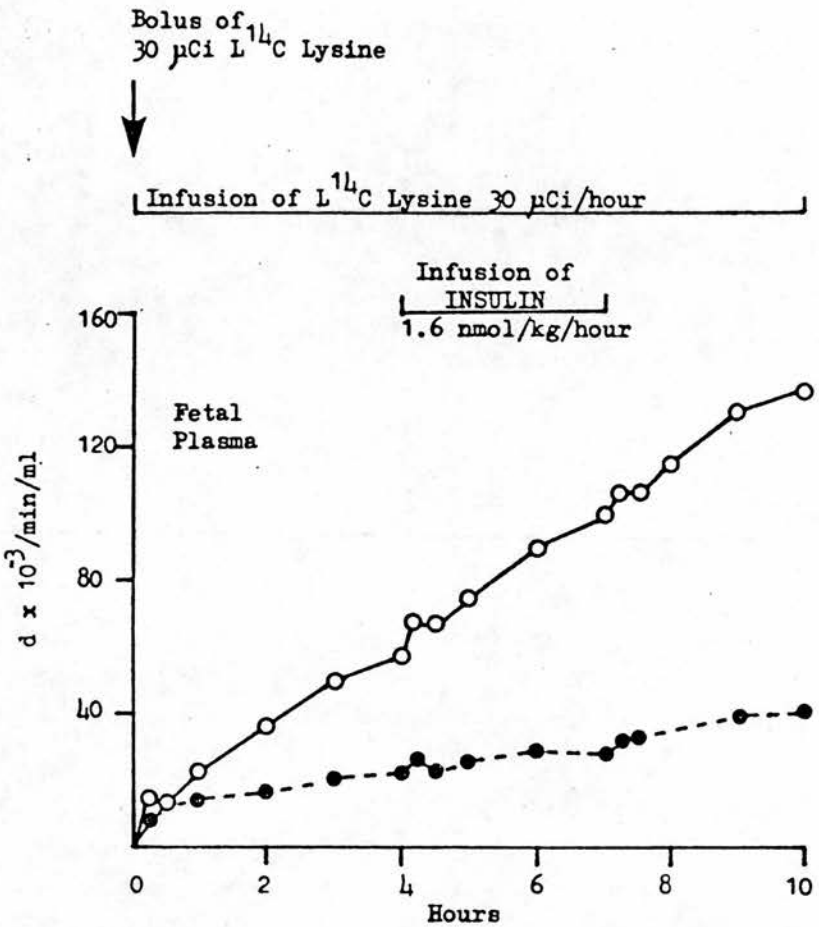


Fig 13ib

Fetal plasma total (0) and deproteinised (●) activities during continuous fetal infusion of $L^{14}C$ lysine (30 µ Ci/hr) and infusion of insulin between hours 4 and 7.

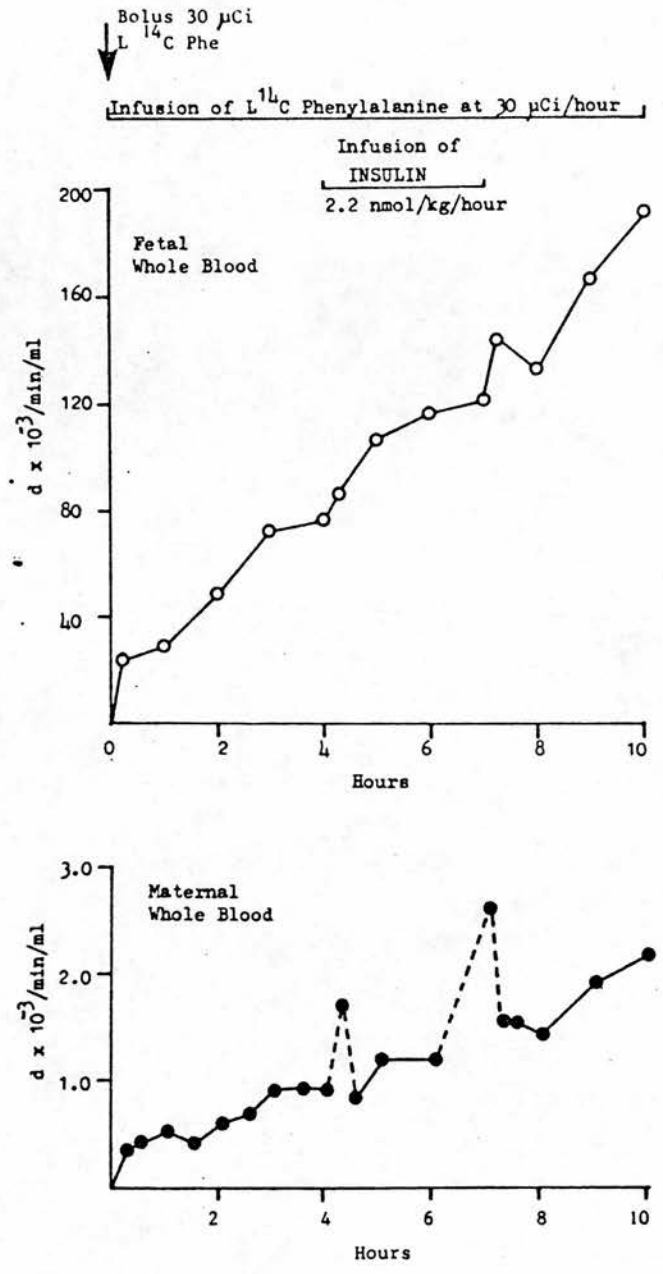


Fig II ii

Maternal (●) and fetal (0) whole blood activities during continuous fetal infusion of L ^{14}C phenylalanine (30 µ Ci/hr) and insulin infusion between hours 4 and 7.

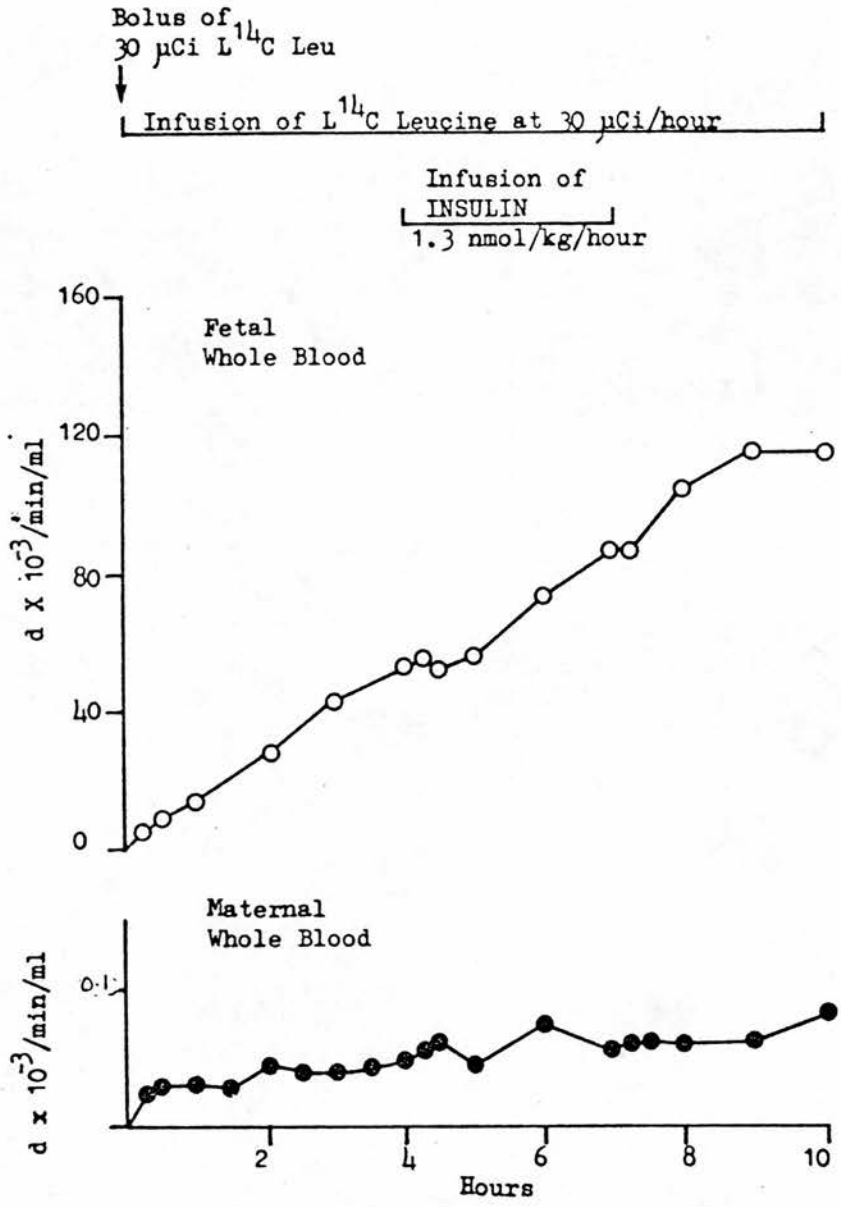


Fig 12ii

Maternal (●) and fetal (0) whole blood activities during continuous fetal infusion of $L^{14}C$ leucine (30 μ Ci/hr) and insulin infusion between hours 4 and 7.

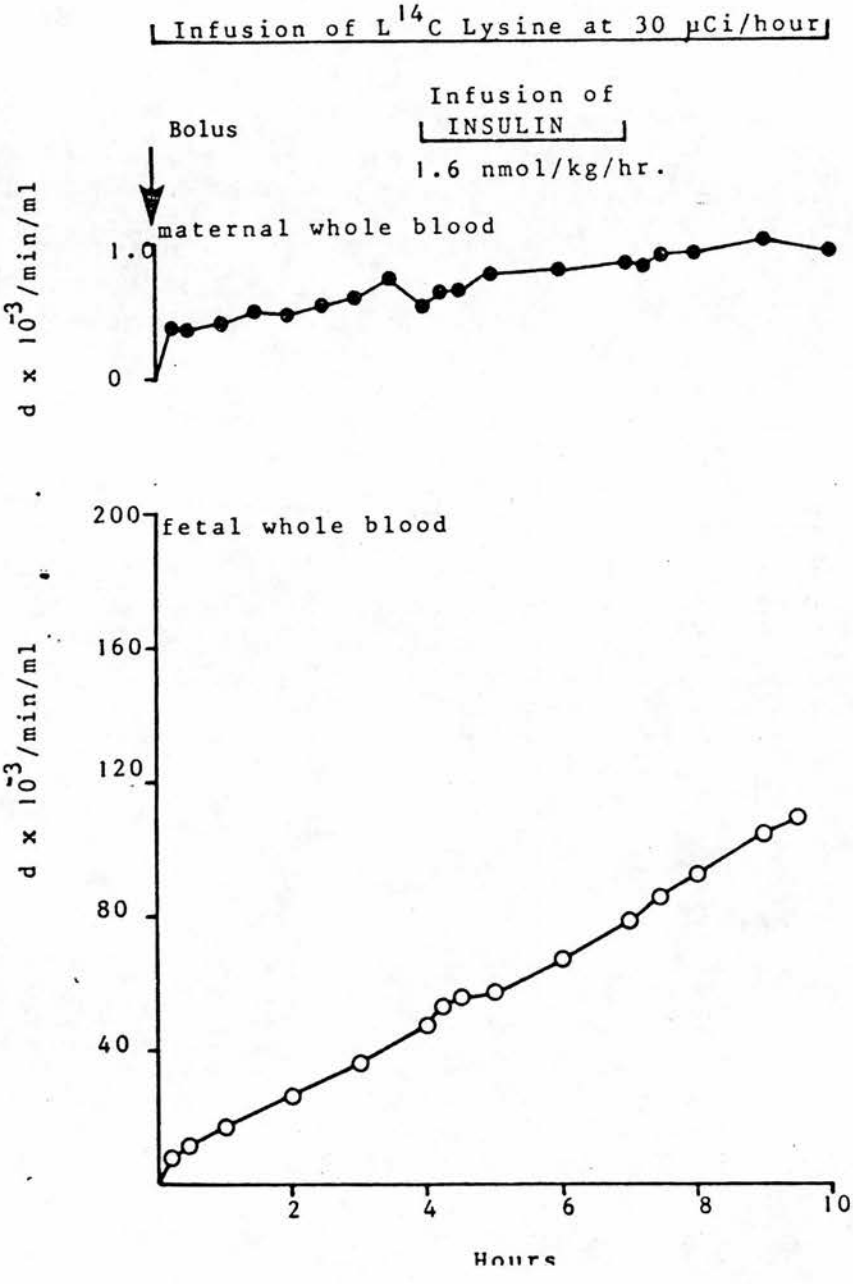


Fig 13ii.

Maternal (●) and fetal (0) whole blood activities during continuous fetal infusion of $L^{14}C$ lysine (30 µ Ci/hr) and insulin between hours 4 and7.

c. Total and Deproteinised Plasma Radioactivity Measurements

Figs 11i a and b, 12i a and b, 13i a and b.

Maternal total plasma activities were low, and the radioactivity of the deproteinised plasma was too low for accurate measurement. The total activity increased slowly in a linear manner with only small deviations occurring when insulin stopped in experiments Ins C2 and Ins C4.

Although the radioactivity was greater in fetal samples, like maternal samples there was no significant change in the rate of incorporation (i.e. in total plasma) when insulin was either introduced or withdrawn. Similarly although the deproteinised plasma radioactivity showed greater variability in its rate of increase, insulin was not associated with any significant changes.

d. Radioactivity in Whole Blood

Figs 11ii, 12ii, 13ii.

Radioactivity in maternal whole blood was low. Only in experiment Ins C2 (phenylalanine) was insulin associated with a rapid change in the rate of increase of activity, a peak at $4\frac{1}{4}$ hours and a rapid fall from a peak at 7 hours occurring. The fall in radioactivity between 7 and $7\frac{1}{4}$ hours was also observed in total plasma. These maternal 'peaks' in activity coincide with peaks in the fetal haematocrit - 54% at $4\frac{1}{4}$ hours and 52% at 7 hours. Radioactivity in fetal whole blood however failed to show any response to infusion of insulin or the changed haematocrit at $4\frac{1}{4}$ hours, and only a moderate increase in radioactivity was observed on stopping insulin. No increase was observed at the 7 hour haematocrit peak. Fetal whole blood radioactivity of the other 2 fetuses was unaffected by the infusion of insulin.

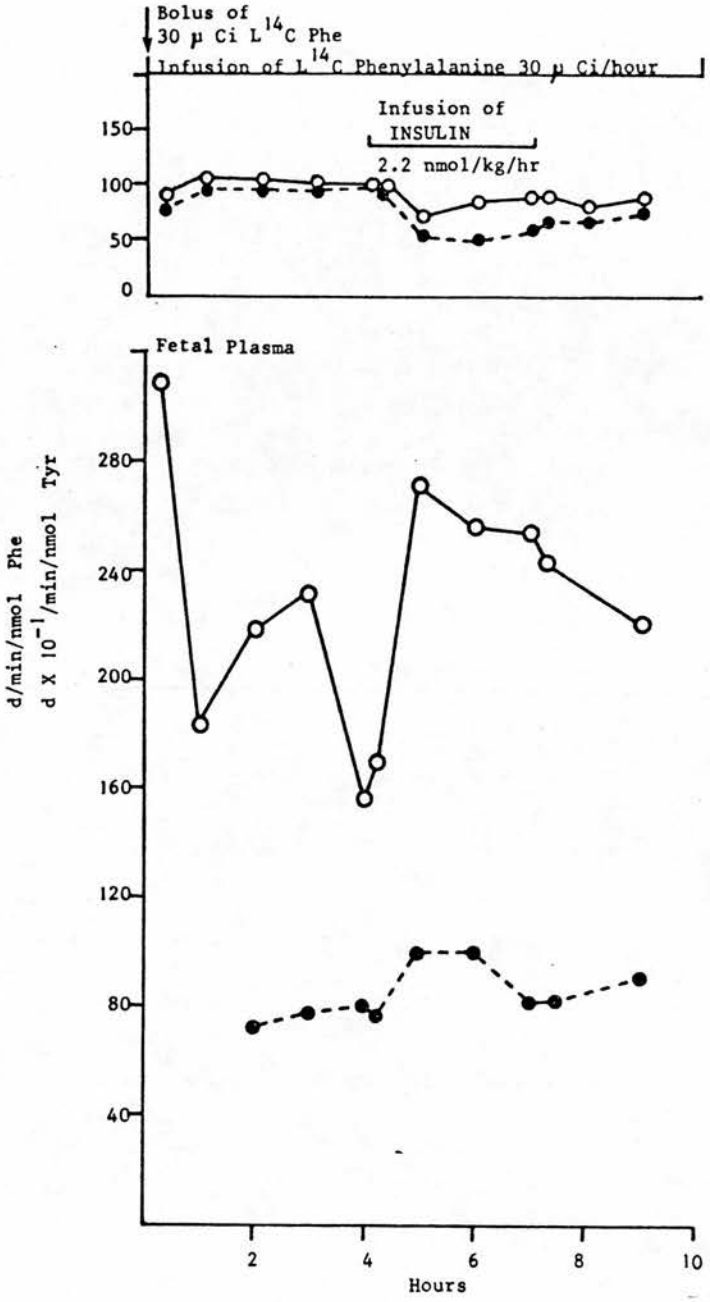


Fig Hiii

Fetal plasma water phenylalanine (0) and tyrosine (●) SA (below) and concentration (above) during continuous fetal infusion of L ^{14}C phenylalanine (30 μ Ci/hr) and insulin infusion between hours 4 and 7.

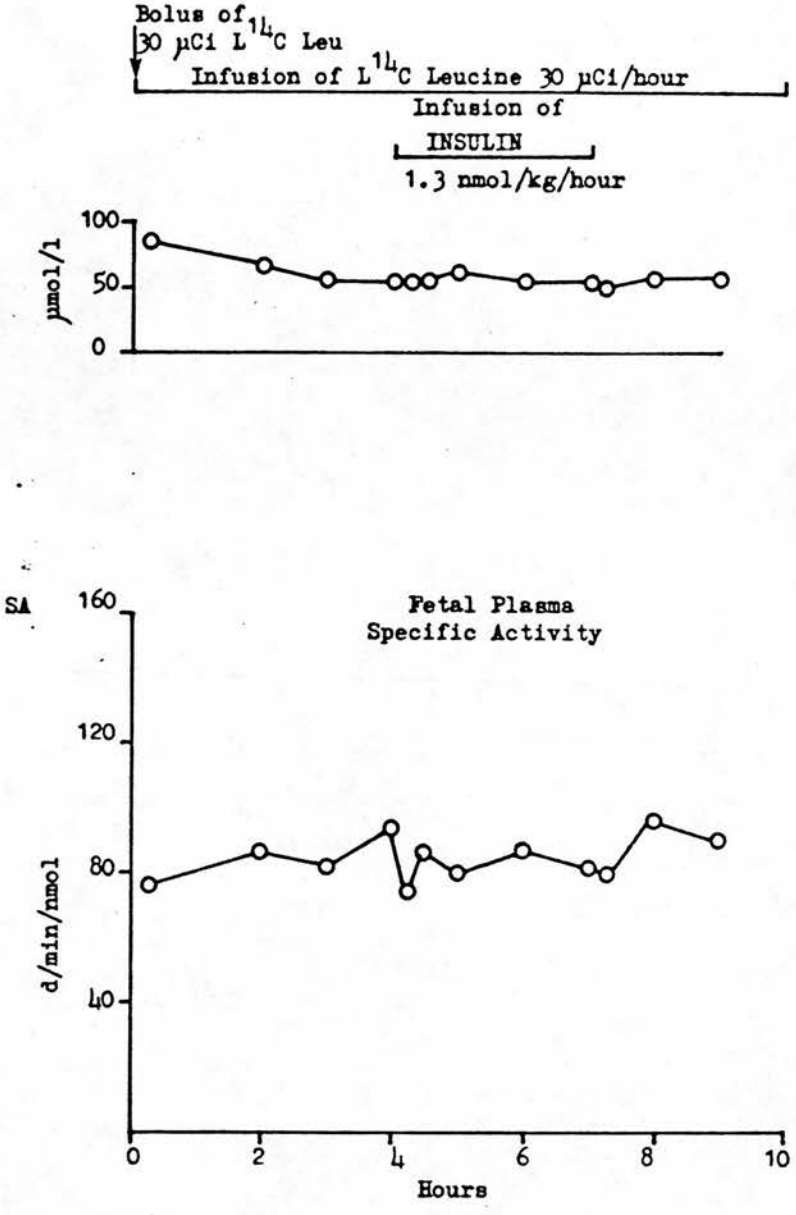


Fig 12iii

Fetal plasma water leucine SA (below) and concentration (above) during continuous fetal $L^{14}C$ leucine infusion (30 µ Ci/hr) and insulin infusion between hours 4 and 7.

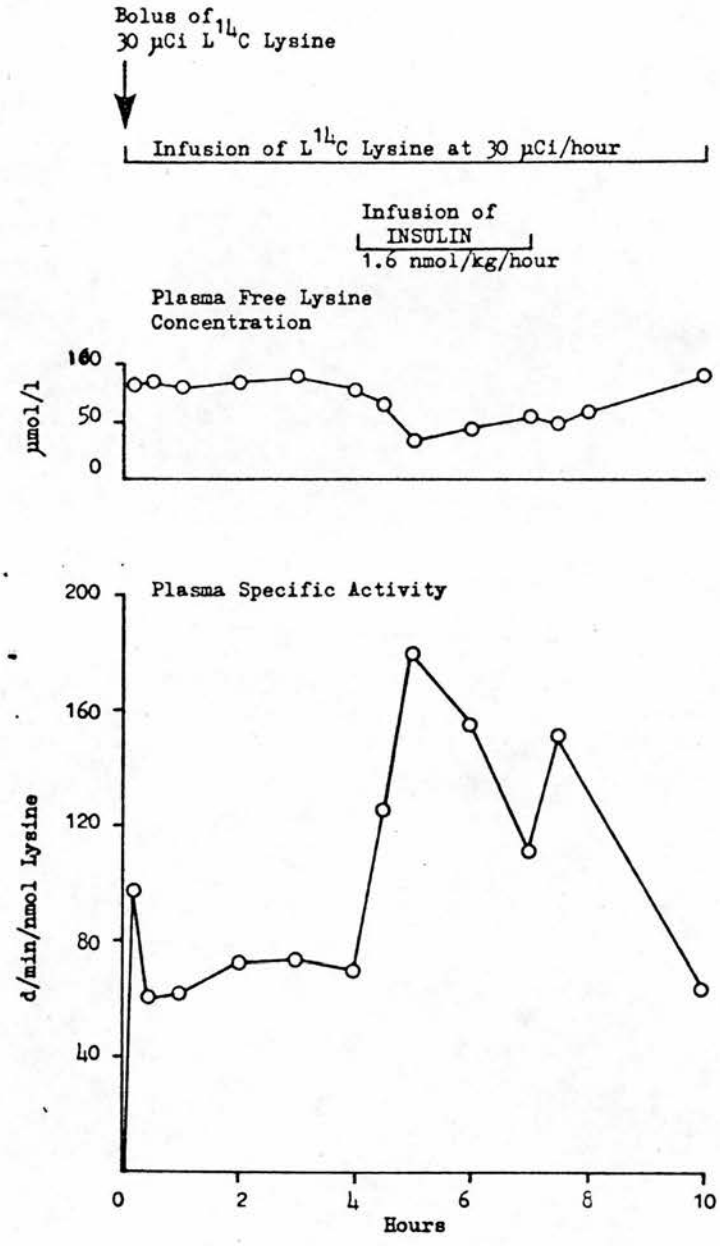


Fig 13iii

Fetal plasma water lysine SA (below) and concentration (above) during continuous fetal infusion of $L^{14}C$ lysine (30 μ Ci/hr) and infusion of insulin between hours 4 and 7.

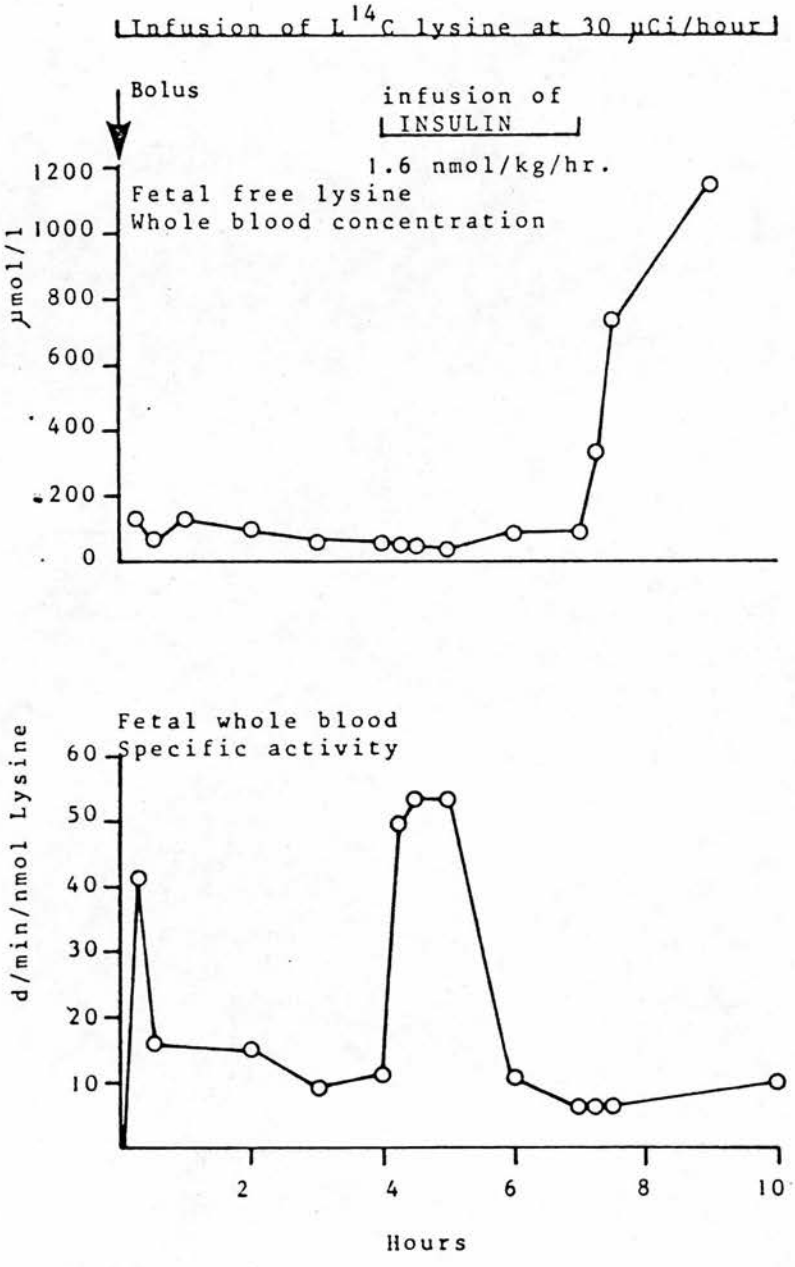


Fig 13iv

Fetal whole blood free lysine SA (below) and concentration (above) during continuous fetal infusion of $L^{14}C$ lysine (30 μ Ci/hr) and infusion of insulin between hours 4 and 7.

e. Specific Activity Measurements

Figs 11iii, 12iii, 13iii and iv

The SA of phenylalanine and lysine in plasma water of the fetuses infused with the respective amino acid was changed significantly when insulin was infused. However insulin had no effect on the leucine SA of the fetus infused with that amino acid.

Both free phenylalanine and lysine plasma concentrations decreased on infusing insulin but the concentration of leucine remained constant, its only decrease being earlier during the 4 hour basal period at a time when fetal haematocrits fell from 48.5 to 42%. The decrease in plasma phenylalanine and lysine concentrations was associated with an increase in SA. However, in the phenylalanine infused fetus SA had fallen (duplicate analyses) prior to the time at which insulin infusion was thought to have begun (See page 176). Tyrosine did not decrease, but did increase from 4 to 5 hours. From 5 hours onwards the phenylalanine and lysine SA returned towards pre insulin values, withdrawing insulin having little affect on the phenylalanine infused fetus and producing only a small temporary increase in lysine SA.

The SA of free lysine in whole blood, although increasing rapidly on infusing insulin had already returned to pre-insulin values by hour 7 when insulin was withdrawn. SA was unaltered at this time but the free plasma lysine concentration increased markedly reaching 1150 μmol/l at 10 hours.

f. Flux

Fetal phenylalanine flux was calculated at 137μmol/Kg/hr, leucine flux at 217.3 μmol/Kg/hr and lysine flux at 318 μmol/Kg/hr.

Experiment Ins C5

Fetal Infusion LU^{14}C Lysine and Insulin

Although there was some doubt, it was thought that the umbilical catheters were correctly sited. However, it had not been possible to obtain fetal blood samples from the umbilical arterial catheter for some days although it was still possible to flush the catheter. For this reason infusion was into the "umbilical arterial" catheter and samples were withdrawn from the fetal venous catheter. It was later discovered that infusion had been into the amniotic sac and the experiment was repeated (experiment Ins C4). Infusion of 330 μ Ci LU^{14}C lysine, under conditions identical to experiments Ins C2 and Ins C3, was commenced at 22.01 hours and infusion of insulin at 02.00 hours. Insulin was stopped at 05.00 hours and the infusion at 08.06 hours. Maternal and fetal blood samples were taken as in experiment Ins C2. Blood samples for glucose estimations were taken into fluoride tubes at 2,4,6,7,8,9, and 10 hours.

Results

a. Haematocrit

Maternal values were all within the range 28 - 31 per cent and fetal values between 41 and 44 per cent.

b. Blood Glucose Values

TABLE VII

Time Hours	Plasma Glucose * mmol/l		Insulin ng/ml	
	Maternal	Fetal	Maternal	Fetal
0	-	-	-	0.59
2	0.67	0.44	-	-
4	0.70	0.25	-	0.51
4½	-	-	-	-
6	0.63	ND	-	-
7	0.54	0.10	0.43	0.82
7½	-	-	-	-
8	0.51	0.59	-	-
9	1.09	0.44	-	-
10	0.39	0.25	0.72	0.56

* All samples precipitated with 3 per cent perchloric acid.

From the table it can be seen that fetal plasma glucose decreased during insulin infusion but fluctuation with low concentrations were also recorded in periods off insulin. Maternal glucose was unaffected by fetal insulin infusion, fluctuations being present throughout the experiment. No correlation between maternal and fetal glucose was found.

ND - non detected

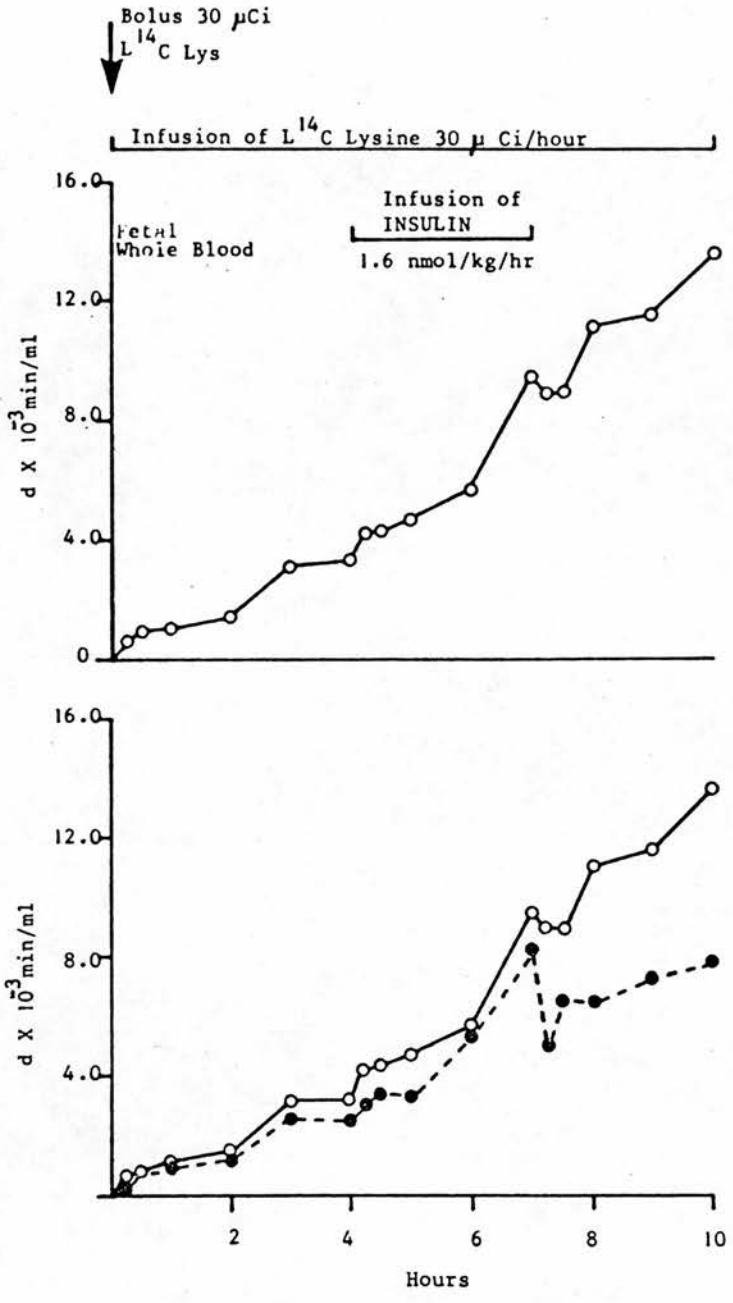


Fig 14i

Fetal whole blood (above) and plasma total (O) and deproteinised (●) activities (below) during $L^{14}C$ lysine infusion into amniotic fluid, with insulin infusion between hours 4 and 7.

c. Insulin

Measurement of insulin concentration confirmed that no insulin had been infused into the fetal blood compartment, little change being noted throughout the experiment,

d. Maternal Total Plasma and Maternal Deproteinised Plasma

Levels of ^{14}C activity were too low to measure.

e Eetal Total Plasma Fig 14i

Fetal plasma ^{14}C activity gradually increased but the rate of increase was not consistent for any extended period of time

The same pattern of increasing activity was found in the plasma water as in total plasma. The decrease between 7 and 7½ hours observed in total activity was more exaggerated in plasma water and from this time the percentage of total activity present in the plasma water fraction was less.

f. Whole Blood ^{14}C Activity Fig 14i

Maternal levels were too low to be detected above background. No consistent rate of increase was observed in fetal samples.

g. Tissue Digestion

Radioactivity measurements of the placental tissue are given

In Part V.

TABLE XI

INSULIN INFUSION RATES

Experiment	iu/kg/hr	µg/kg/hr	nmol/kg/hr
Ins C1	0.290	12.09	2.1
Ins C2	0.305	12.73	2.2
Ins C3	0.181	7.53	1.3
Ins C4	0.226	9.45	1.6
Ins C5	0.224	9.35	1.6

iu = 0.0417 mg

mw = 5777

EXPERIMENTS ON THE EFFECTS OF INSULIN INFUSION

Insulin Concentration

The rate of insulin infusion for these experiments was calculated at 1.44 nmol (0.2iu or 8.33 ug/kg/hr) based on a fetal weight of 3 kg - the expected weight of the fetuses (which were thought to be smaller than those of the previous year - 4 kg). At sacrifice, the fetuses were weighed and actual infusion rates were 2.1, 2.2, 1.3, 1.6, and 1.6 nmol/kg/hr for experiments Ins C1, Ins C2, Ins C3, Ins C4 and Ins C5 respectively. (See opposite for infusion rate iu and µg/kg/hr.) With the exception of the Ins C3, in which insulin was infused into the amniotic fluid, plasma insulin concentrations were increased between 7 and 55 fold above the initial concentration. The rate of increase was variable, and although the concentration of plasma insulin decreased immediately on stopping the insulin infusion in all experiments, the rate of decline also varied.

It should be noted that fetal plasma of experiment Ins C2 also appeared to have a considerable increase in insulin concentration from 0.57 to 6.12 ng/ml before the infusion of insulin was thought to have commenced with only a small initial increase in insulin concentration occurring following the introduction of insulin. It is not possible to determine retrospectively whether this increase was due to endogenous insulin secretion or to accidental premature introduction of insulin into the fetus.

Activity Measurement

Infusion of insulin had almost no effect on the rate of accummulation of radioactivity in the plasma and whole blood of both the fetus and mother, any changes being only minor and temporary. SA measurements would be necessary to assess the effect on the rate of protein synthesis:- a decreased SA with an increased rate of synthesis would obscure any effect in total radioactivity measurements.

Influence of Insulin on Specific Activity

In the experiment Ins C2 (phenylalanine infusion into the fetus) there was a twelve fold increase of insulin in the 4 hour fetal blood sample compared with the initial insulin concentration. Whether this is of endogenous or exogenous origin should not influence the fetal response, if any, to this increased concentration and therefore changes resulting from the affect of insulin on metabolism should be apparent at 4 hours. If the magnitude of response is related to either the absolute concentration of insulin or degree of change the greatest change should be observed in this experiment in which both the highest concentration was reached and the greatest fluctuations induced.

In the Ins C2 experiment, as the concentration of insulin increased the phenylalanine S.A. in plasma fell from 250 d/min/nmol to 160 d/min/nmol. As both input of labelled amino acid and plasma free amino acid concentrations were constant during this initial period of insulin infusion this reduction in S.A. must be the result of dilution with unlabelled amino acids. From the decreased S A and constant amino acid con-

centrations it may also be inferred that the entry and exit rate of free amino acids into and from the plasma pool are balanced, but that the two rates (i.e. turnover) are increased by equal amounts. However, between 0.25 hr and 1 hour after commencing the insulin infusion the free amino acid plasma concentration of phenylalanine decreased from 100 to 60 μmol/l and tyrosine from 100 to 55 μmol/l. Small changes in concentration could be a result of fluctuations in the plasma and blood pool volumes or haematocrit. However, it is unlikely that the changes of 40 to 50 per cent observed in this experiment would be accounted for solely by such fluctuations over a period of 45 minutes, particularly as the haematocrit remained constant during this time (Appendix 6). One hour post insulin infusion, the concentration of amino acids decreased and the S-A of phenylalanine and tyrosine increased to levels slightly higher than those pre insulin. This would indicate that the rate of removal of free amino acids from the plasma pool had increased, and that this increase was not even partially compensated for by increased entry of unlabelled amino acid.

As insulin has been shown to increase cellular uptake of amino acids (chapter 1, 6) it is unlikely that the unlabelled amino acids entering the plasma pool were from fetal tissue pools, but more likely they were of maternal origin, increased placental transfer of the unlabelled amino acids producing the initial pool dilution.

Changes in placental transfer rates with changing fetal insulin concentration are probably mediated through altered amino acid concentration gradients between fetal blood, the syncytiotrophoblast and maternal blood. Placental parenchyma has a high concentration of amino acids[67]. It is thought that amino acids are actively taken up from the maternal circulation into the placental syncytiotrophoblast, from which they are released through cytotrophoblast into fetal blood by passive diffusion down a concentration gradient. If fetal tissues respond to insulin in a manner similar to that of adult tissues (see page 45) cellular uptake of amino acids would increase, thereby reducing the plasma free amino acid concentration. Lower fetal plasma amino acid concentrations would thus stimulate transfer by increasing the blood: placenta amino acid concentration gradient and in fetal infusions this would be detected by decreased S A - as seen 15 minutes after the start of insulin infusion in experiments Ins C2 and Ins C4.

Insulin might also increase amino acid uptake into fetal placental cells. Thus, unless placental protein synthesis is sufficiently active to utilize the incoming amino acids, the difference between fetal blood and placental intracellular amino acid concentrations might be increased even more. However, the increased S.A and decreased plasma amino acid concentrations in fetal infusions (Ins C2 and Ins C4) from 15 minutes to one hour after insulin had commenced, suggests that the promotional effect of insulin on cellular uptake is more powerful than the passive diffusion mechanism by which amino acids are released into fetal blood, the inverse relationship between fetal plasma amino acid concentration and placental

transfer disappearing. If the effect of insulin on uptake of amino acids only related to the fetal margin of placental cells - only insulin receptors on this side of the cell being exposed to the infused insulin - uptake from adjacent maternal blood cell and plasma might also be compromised. Decreased transport between fetal cells of the trophoblast and maternal blood might therefore occur - a raised fetal cellular amino acid concentrations increasing the gradient up which amino acids must be transferred. At one hour post insulin the plasma S.A values returned to pre insulin levels. Similarly, from one hour onwards free plasma amino acid concentrations slowly increased toward the pre insulin concentrations. An increase in the rate of placental transfer of cold amino acids would decrease the S A and increase the plasma concentration. However if this were true the S.A would be lower than the pre insulin value if amino acids were continuing to enter cells at an accelerated rate under the influence of insulin. Thus these changes suggest that although the concentration of insulin was still increasing the effect of insulin was diminishing and cellular uptake from free amino acid pool was decreasing towards pre insulin rates. The end organ response to insulin may therefore be limited - and may be more sensitive to rapid change in insulin concentration rather than actual concentration. Additionally the decreased blood glucose concentrations from 8.9 to 1.7 mmol/l in Ins C1 and from 7.1 mmol/l to ND.* in Ins C4 may limit the uptake of amino acids into cells. This may not only lead to an increase in the plasma concentration but also allow increased diffusion of amino acids from the placenta to fetal blood. Increased placental protein

* ND no detected glucose

synthesis because of either the effect of insulin or high amino acid concentrations could also lower the gradient between intracellular concentration of fetal placental cells and maternal blood. Withdrawing insulin did not alter the rate of decrease in phenylalanine S A suggesting that the influence of insulin on fetal amino acid metabolism and placental transfer was already diminishing.

During insulin infusion the ratio of tyrosine:phenylalanine concentration changed from 0.90 to 0.64 returning to 0.88 at 8 hours - 1 hour after insulin infusion stopped. If phenylalanine had been hydroxylated in the placenta or fetal liver (see page 136) this could reflect increased uptake of phenylalanine and tyrosine but reduced release of the metabolised phenylalanine i.e. tyrosine etc. There was little initial change in tyrosine S A on infusing insulin at the time when increased quantities of cold phenylalanine entered the plasma. This would suggest placental hydroxylation with release of prelabelled tyrosine - the S A only increasing when the tyrosine concentration fell. The fall in S A occurred later (6 - 7 hours) than that in phenylalanine S A (5 - 6 hours) and again corresponded to the start of amino acid concentration recovery. On stopping insulin, unlike phenylalanine S A the tyrosine S A increased - possibly reflecting release of prelabelled intracellular tyrosine as the influence of insulin diminishes.

Although the timing of events was a little different, plasma changes induced by insulin in experiment Ins C4 - lysine infusion into the fetus-resembled those of Ins C2.

After 1 hour of insulin infusion, both whole blood and plasma lysine SA decreased. At this time although the plasma free lysine concentration was increasing, in whole blood the concentration was unchanged. This would imply that the effect of insulin was similar to that in the phenylalanine infusion, and diminished with time.

Withdrawing insulin resulted in a small temporary increase in plasma lysine SA. This would be compatible with insulin stimulation of the formation of a membrane amino acid complex, which would disocciate to some extent as insulin concentration decreased, releasing previously bound labelled amino acids into plasma and probably cell cytoplasm.

In whole blood although free lysine concentration increased rapidly after discontinuing insulin there was little change in lysine SA. Amino acids must therefore be entering from a prelabelled source. Release of amino acid from a small pool with rapid turnover could increase concentration whilst maintaining SA. Alternatively, binding of released tissue amino acids to the RBC could increase the concentration of whole blood free amino acids on precipitating with acid.

Thus, insulin concentration appeared to have some influence on size and flux between amino acid pools, increasing the fetal cellular and thereby decreasing the plasma and blood pool. The concentration of insulin in this experiment was less and the percentage increase smaller than in experiment Ins C2 and could explain some of the differences found. However, as the S A increased in both fetuses while concentration decreased, in neither instance was the transfer rate of amino acids across the placenta able to keep pace with the entry rate of amino acids into tissues, although initial dilution with unlabelled amino acid occurred in the plasma of Ins C2.

This contrasts with the findings in the fetus infused with leucine, in which there was little response to insulin, both plasma free amino acids and SA being relatively unchanged. This could be a result of the smaller increment in insulin concentration, as in both the phenylalanine and lysine infused fetuses the effect of insulin diminished as the rate of increment decreased. Even if the rate of insulin infusion were diminishing because of absorption of insulin to either the syringe or infusion line, as the plasma concentration of insulin continued to increase throughout the experiments, the effect of insulin should be unaffected if it is concentration alone which determines the response of the fetus to insulin. The lower insulin concentration would probably have reduced the additional requirement for glucose so that in the leucine infused fetus, fetal blood glucose concentrations remained relatively constant. In contrast in both Ins C2 and Ins C4 fetal blood glucose concentrations were decreased during the infusion of insulin and in Ins C4 maternal glucose was significantly reduced between hours 6 and 7. Thus in Ins C3 either placental glucose transfer and/or fetal gluconeogenesis was accelerated sufficiently to meet the increased glucose requirement on infusing insulin, or the end organ response was depressed and fetal metabolism was relatively more resistant to insulin induced changes. Of these factors the observed decrease in maternal glucose could suggest an increased placental transfer. The greater weight of this fetus (3.650 kg) of the same gestational age as the other fetuses infused with insulin - 2.160, 2.940, 2.274 and 2.910 kg could suggest a higher mean placental transfer of glucose during pregnancy. As blood glucose did not decrease it is unlikely BCAA oxidation increased in muscle, although a glucose sparing action of increased oxidation cannot be excluded.

The differences in blood glucose concentrations are unlikely to account for the differences in SA changes between the leucine and phenylalanine or lysine infused fetuses. Infusion of glucose together with insulin failed to alter the effect of insulin on protein synthesis in fetal lambs.[337] Additionally, in these experiments the greatest changes in plasma amino acid concentration and SA were at a time before glucose concentrations decreased. The diminishing effect of insulin could however be related to lack of energy for protein synthesis or intracellular transport, although the requirement for glucose has been shown to be only for transport and synthesis, stimulation of these by insulin being independent of glucose concentration.[285,286]

Alternatively, utilization of phenylalanin eand lysine in gluconeogenesis in the latter part of the insulin infusion could have occurred.

If, as in adults glucose homeostasis is controlled by hepatic tissue (the placenta is also important in the fetus) preferential uptake of leucine by muscle may be less affected than the extraction of amino acids by liver on infusing insulin.[230,231]

However, in the adult sheep[290] insulin was found to regulate gluconeogenesis by its influence on muscle metabolism and had little effect on the hepatic extraction of amino acids. Fetal metabolism or increased glucagon secretion could lead to increased amino acid extraction by the liver when glucose concentrations are low. Also differences between the behaviour of leucine and other amino acids could occur if hepatic tissue were more sensitive than muscle to changes in plasma insulin concentration. Any difference between hepatic and muscle cellular uptake could also be exaggerated by the infusion of both insulin and labelled amino acids into the umbilical venous blood of which 58 - 67% passes directly through the liver before entering the general circulation.[307,308]

The shorter half life of hepatic proteins - 0.75 days compared with 1.08 days of mixed proteins in muscle of fetal lambs[336] would also exaggerate any difference in absolute terms (though not necessarily percentage values), in the incorporation of amino acids into tissue proteins, and therefore in amino acid utilization.

Hepatic tissue did have the greatest uptake of activity (represented by the remaining activity in the tissues at sacrifice - (see page 190), incorporation into protein being approximately 2 - 3 times that of muscle in experiments C1 - C4 and 4.5 times that of muscle in Ins C4 (lysine infusion with insulin). Soltesz[339], in newborn lambs also found liver to have the greatest bound leucine activity, the liver:muscle S A ratio being 2.34, but free leucine S A was greater in muscle. Leucine oxidation occurs in muscle but not brain and liver[242,243] and this could also partially explain the different response to insulin.

Differences between phenylalanine and lysine infusion and that of leucine could therefore be a result of individual variation, insulin concentration or tissue sensitivity, or the different metabolism of the amino acids.

If infusion of insulin into the fetus increases placental transfer of amino acids, the removal rate from maternal blood would also be expected to increase. Thus maternal plasma amino acid concentrations should decrease and the S A of maternal amino acids would be expected to increase in the $L^{14}C$ lysine maternal infusion during the period of insulin infusion into the fetus, although the larger pool of amino acids in maternal

plasma would reduce the magnitude of change. The concentration of maternal plasma free lysine did decrease from 118.6 μmol/l 15 minutes post insulin to 69.6 μmol/l one hour post insulin, the S A increasing from 30.8 d/min/nmol to 54.7 d/min/nmol during this time. However further fluctuations of both concentration and S A were observed during the remaining two hours of insulin infusion. Fluctuations could be those normally occurring but could reflect the diminishing effect of insulin on fetal metabolism, with consequent alterations in fetal demand. Changing fetal demand would alter placental transfer rates and thereby the demands on the maternal supply, catabolism of maternal protein subsequently altering to meet the changed conditions. Greatest fluctuations were observed during insulin infusion with the exception of the rapid increase in lysine concentration and decrease in S A in the first 15 minutes after stopping the fetal infusion of insulin - a time at which fetal concentrations and S A of Ins C2 and Ins C4 indicated placental transfer to be temporarily diminished. The proportion of plasma water activity associated with lysine also decreased post insulin, suggesting there was an increased degradation in place of immediate transfer of amino acids to the fetus. A sudden decreased placental transfer rate could also be expected to increase maternal concentration and decrease lysine S A if unlabelled amino acids, resulting from maternal catabolism, continue to enter the plasma at the same rate. Thus, even though the proportion of infused ^{14}C lysine transferred to the fetus was less there could be an increased dilution of the labelled lysine and lower S A

Conclusions on the Effect of Insulin on Placental Transfer

1. Insulin decreased fetal blood glucose in three of four fetuses, Maternal glucose decreased slightly in two and markedly in one ewe.
2. In the two fetal infusions in which blood glucose decreased the plasma concentrations of phenylalanine and lysine - the $L^{14}C$ amino acids infused - decreased and the S.A increased during the first hour of insulin infusion. This suggested fetal cellular uptake was increased.
3. In the same two fetuses the increased S A was not maintained, and the plasma amino acid concentrations returned towards preinsulin values, suggesting that the effect of insulin was only temporary.
4. In the fetus infused with leucine, maternal glucose decreased but there was no change in either plasma glucose or leucine S A Therefore either utilization of lysine and phenylalanine for energy production was increased, the effect of insulin on placental transfer and cellular uptake of leucine was different or the individual response varies.
5. Fetal infusion of insulin with maternal infusion of lysine indicated placental transfer increased during the first hour but thereafterwards fluctuated. Highest plasma lysine concentration and lowest S A were recorded 15 minutes after terminating the insulin infusion.

6. Whole blood S A of Ins C4 suggested the cellular components of blood - probably the erythrocyte - were important in transfer of amino acids and that this was influenced by insulin. The rapid increase in S.A without any increase in concentration on starting and the rapid increase in concentration with no affect on S.A on stopping insulin suggests binding and release of amino acids to the RBC may occur rapidly, and on stopping insulin release of amino acids or breakdown of peptides, preaccumulated under the influence of insulin, could occur.

Future Experiments

Further research should include the following:-

1. Investigation of the role of the RBC in transport, and the effect of insulin on this.
2. The effect of insulin on transport with maintainance of blood glucose concentrations.
3. Investigation of the uptake and transfer of leucine by simultaneous infusion of $L^{14}C$ leucine and either $L^{14}C$ lysine, $L^{14}C$ phenylalanine or other essential amino acid.
4. Infusion of $L^{14}C$ alanine and the influence of insulin on placental transfer with and without additional glucose.

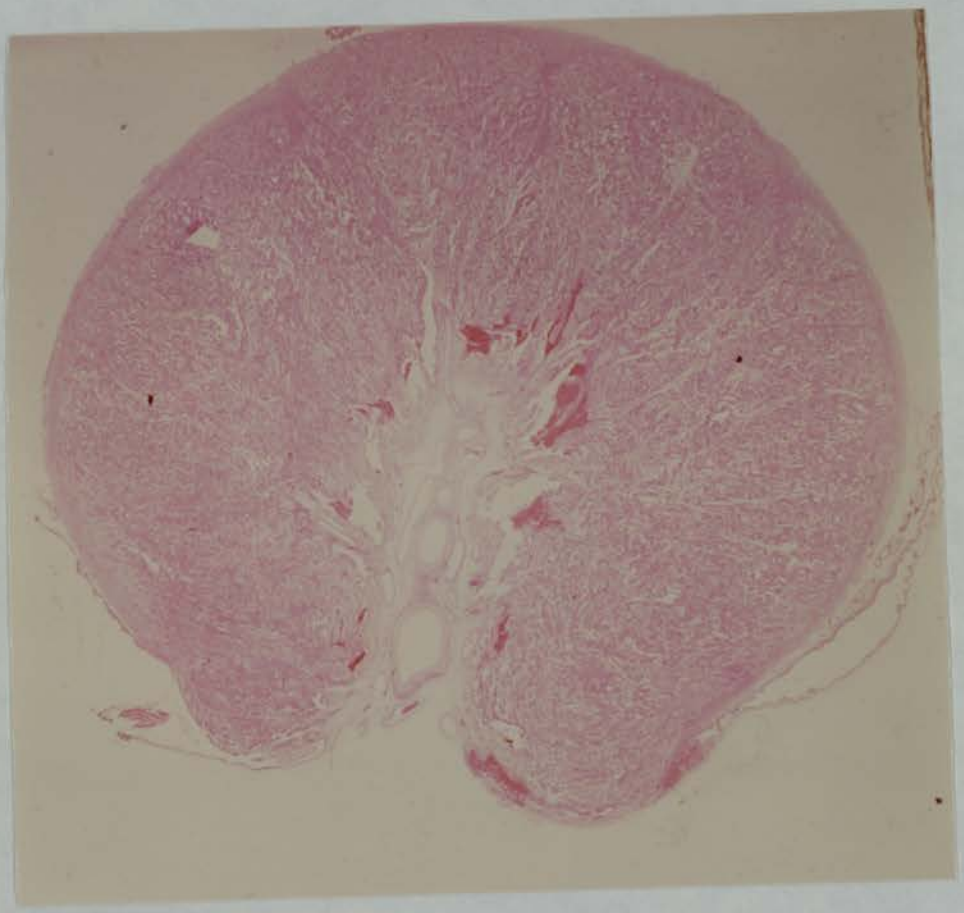


Fig 15

Structure of Sheep Placental Cotyledon

Autoradiographic Tissue section stained with haematoxylin and eosin.

Part V

Autoradiography

Although autoradiography was employed to study the distribution of activity within tissues, no additional information to that obtained from tissue digestion and radioactivity counting was obtained. Activity, represented by dark granules on the tissue slides, was generally distributed throughout the tissues including that of the placenta, and no zones of activity concentration were found. This may have been expected in experiments C1 to C4 in which tissue was only obtained 7 days after the labelled infusion, but there was no significant difference between the distribution of activity in these experiments and that of Ins C4 from which tissues were obtained immediately.

A section of a placental cotyledon is shown in Fig 15 Although little activity can be recognised in this, it illustrates the vascular fetal core of the cotyledon, with the denser maternal tissue around the outer zone.

Also no difference in the distribution of activity within the placenta was noted between maternal (Ins C1) and fetal (Ins C4) infusions from which tissue was taken at the end of infusion.

The relatively low activity of these tissues to that normally present in tissues prepared for autoradiography may have prevented observation of small differences in distribution.

Tissue Activity and Autoradiography

Tissue Counting

The ^{14}C activity of fetal tissues from experiments C1, C3,

TABLE XII

Radioactivity in tissues (d X 10^{-3}/min/g) and ratio tissue:maternal placental activity (T:MP)

	Experiment C1		Experiment C2		Experiment C3	
Fetal Tissue	d x 10^{-3}/min/g	Ratio T:MP Activity	d x 10^{-3}min/g	Ratio T:MP Activity	d x 10^{-3}/min/g	Ratio T:MP Activity
Adrenal	35.3	3.0	27.3	1.7	188.7	5.3
Brain	15.2	1.3	19.4	1.2	89.5	2.5
Heart	27.9	2.4	28.7	1.7	178.7	5.0
Kidney cortex	26.6	2.3	24.9	1.5	169.1	4.7
Kidney medulla	20.0	1.7	14.9	0.9	97.9	2.7
Liver	38.5	3.3	33.7	2.0	221.6	6.2
Lung	16.2	1.4	16.4	1.0	99.6	2.8
Muscle	18.9	1.6	20.0	1.2	109.8	3.1
Fetal Placenta	29.9	2.5	23.4	1.4	74.2	2.1
Maternal Placenta	11.8	1.0	16.5	1.0	35.9	1.0
Spleen	37.9	3.2	37.1	2.2	234.9	6.5
Thymus	23.1	2.0	26.8	1.6	121.5	3.4
Thyroid	49.5	4.2	25.5	1.5	244.0	6.8

TABLE XII.(cont'd)

Radioactivity in tissues (d X 10^{-3}/min/g) and ratio tissue:maternal placental activity (T:MP)

Fetal Tissue	Experiment C4					Experiment Ins C4		
	d x 10^{-3}/min/g		Ratio T:M Activity		Ratio	d X 10^{-3}/min/g	Ratio T:MP Activity	Maternal d X 10^{-3}/min/g
	^{3}H	^{14}C	^{3}H	^{14}C	$^{14}C:^{3}H$			
Adrenal	159.5	18.1	3.6	3.0	8.8			
Brain	71.0	7.5	1.6	1.3	9.5			
Heart	132.0	15.0	2.9	2.5	8.8			
Kidney cortex	119.2	9.0	2.7	1.5	13.2	408.2	12.7	4.5
Kidney medulla	85.8	9.7	9.9	1.7	8.8	193.1	6.0	2.4
Liver	175.4	20.0	3.9	3.4	8.8	832.0	25.8	3.0
Lung	70.5	7.7	1.6	1.3	9.1			
Muscle	82.9	8.8	1.9	1.5	9.4			
Fetal Placenta	80.2	9.4	1.8	1.6	8.5	425.6	13.2	
Maternal Placenta	44.6	5.9	1.0	1.0	7.6	32.2	1.0	
Spleen	320.1	8.9	7.2	1.5	36.1			
Thymus	82.9	11.2	1.9	1.9	7.4			
Thyroid	123.4	15.4	2.8	2.6	8.0			

(maternal and fetal phenylalanine infusions) C2 (maternal leucine infusions) and C4 (dual phenylalanine infusion) is presented in Table XII, the data being the mean value of duplicate analyses.

From the table it can be seen that in all infusions hepatic and splenic tissue were amongs the tissues of highest recorded activity. However phenylalanine and/or labelled metabolites accumulated to the greatest extent in thyroid tissue. Thyroxine, of which phenylalanine is a precursor, increases in ovine fetal plasma from days 50 - 110 and thereafter remains at constant concentration until day 140. The large accumulation of phenylalanine within the thyroid in these fetuses at 126 and 130 days gestation would imply a continued active synthesis of thyroxine within the gland. Also the fact that the activity accumulated in the thyroid when L ^{14}C leucine was infused was no greater than that found in thymus, adrenal, heart and kidney cortex suggests the high activity in the phenylalanine experiments was not due solely to protein synthesis. Specific activity measurements of thyroxine itself would be necessary to confirm this.

As in leucine infusions, labelled phenylalanine, whether infused into maternal or fetal blood circulations was associated with high activity in fetal tissues of the thymus, adrenal, heart and kidney cortex. The adrenal cortex of the fetus of the maternal infusion was in fact as great as that observed in the thyroid in C1 and greater than that of the thyroid in C4. Brain, lung, muscle and the kidney medulla in all infusions were of lower activity than the more metabolically or physically active tissues. The distribution of ^{3}H and ^{14}C activity in the fetal

TABLE XIII

Maternal and Fetal Placental Activity (d x 10^{-3}/min/g)

Experiment Maternal/Fetal		Tissue weight mg	d X 10^{-3}/min/g	Mean d X 10^{-3}/min/g	Fetal:Maternal Ratio
C1	Fetal	66.9	30.1	29.9	2.5
		94.3	29.7		
	Maternal	139.1	14.6	11.7	
		92.6	8.8		
C2	Fetal	213.5	24.6	23.4	1.4
		211.2	22.2		
	Maternal	121.3	15.9	16.5	
		135.5	17.1		
C3	Fetal	161.4	72.6	74.2	2.1
		125.7	75.7		
	Maternal	124.3	33.2	35.9	
		121.0	38.6		
C4	Fetal ^{3}H	303.3	35.4	40.1	1.8
		156.2	44.8		
	Maternal ^{3}H	160.6	22.0	22.3	
		278.2	22.6		

Fetal ^{14}C	303.3	8.0	9.4	1.6
	156.2	10.9		
Maternal ^{14}C	160.6	5.9	5.9	
	278.2	5.9		

TABLE XLI cont

Experiment		Tissue Weight mg	d X 10^{-3}/min/g	Mean d X 10^{-3}/min/g	Fetal:Maternal Ratio
Ins C1	Fetal	41.9	136.9	131.0	1.1
		25.2	125.1		
	Maternal	28.8	113.8	114.4	
		38.0	115.0		
Ins C2	Fetal	25.9	558.3	528.0	5.5
		21.4	497.7		
	Maternal	28.5	109.5	95.7	
		29.2	81.9		
Ins C3	Fetal	43.6	284.6	289.1	8.5
		25.3	293.7		
	Maternal	57.8	35.5	33.9	
		48.3	32.2		
Ins C4	Fetal	53.8	438.0	425.1	13.2
		36.2	413.1		
	Maternal	32.2	47.3	32.2	
		32.9	17.0		
Ins C5	Fetal	40.8	75.0	74.3	1.1
		37.9	73.5		
	Maternal	37.1	69.2	66.1	
		33.0	63.1		

tissues of the dual isotope infusion were similar - the only exception being that of the spleen, the spleen:maternal placenta tissue activity ratio being 7.16 for ^{3}H and 1.50 for ^{14}C. However, part of this discrepancy may be ascribed to technical difficulties. Because of the high concentration of haemoglobin in this tissue there were problems both with colour quenching and, for ^{3}H chemiluminescence (see page 221). The distribution of ^{3}H was similar to that of the ^{14}C fetal infusion - C3 - although, like maximum plasma activities, the level of activity was lower.

The sheep placenta consists of discrete cotyledons (see Fig 1) with maternal tissue surrounding an inner core of fetal tissue. By removal of inner and outer tissue from a cotyledon analysis of the fetal and maternal components may be made. Results of these analyses are given in Table XIII. Maternal components of the placenta were consistently of low activity but the activity of the fetal placenta differed with the type of infusion - maternal or fetal. During fetal infusion of phenylalanine, activity of the fetal cotyledons was low. However maternal infusion of either leucine or phenylalanine, although resulting in only low activity of maternal placental elements, produced relatively high activity on the fetal side of the placenta if compared with other fetal tissues. This may be seen from the tissue:maternal placenta ratios given in Table XII .

Examination of activity in the fetal and maternal tissue of the placentae from experiments Ins C2 and Ins C4 produced the results shown in Table XIII. From this table it may be seen that the pattern

of activity after 10 hours infusion (which included a period of insulin infusion) was similar to that of previous experiments terminated after several days. Fetal activity was consistently greater than that of maternal components, although some back diffusion did occur. As may be expected, in fetal infusions fetal activity was greater and maternal activity less than in the maternal infusion of lysine Ins C1. That ratio of fetal: maternal placenta tissue activity was therefore higher for fetal infusions - 5.51, 8.53 and 16.6:1 than in maternal infusion - 1.5:1 - reflecting the greater transfer of activity from the maternal to the fetal circulations than vice versa. This ratio (1.5:1) is similar (although the level of activity is higher) to that found in experiment Ins C3 - in which lysine was infused into the amniotic fluid. This might imply that there is a relatively extensive exchange between amniotic fluid and maternal blood and tissues - the ratio being closer to that of maternal than fetal infusions. Thus if the amniotic fluid were to be utilized as a route for in utero nutritional supplementation of fetuses at risk because of maternal vascular or placental problems there could be a large loss of the infused material via this route. The lower ratio of the fetal infusion C3 (2.1:1) and higher ratios of C1 maternal infusion (2.5:1) than the corresponding insulin infusions could be a result of the extended time interval between infusion and sacrifice allowing equilibration between the placental components. However the ratio 1.41:1 of experiment C2 - maternal leucine infusion - is in agreement with Ins C1 ratio 1.5:1 although activity is lower. This suggests that subsequent metabolism of individual amino acids such as phenylalanine might be significant. The relative activity of hepatic, renal and placental tissue from the fetus infused with

TABLE XIV

AMNIOTIC AND ALLANTOIC FLUID AND FETAL URINE ACTIVITY

$d \times 10^{-3}$/min/ml

Experiment	C1	C2	C3	C4		Ins C1	Ins C2	Ins C3	Ins C4	Ins C5
				^{3}H	^{14}C					
Amniotic Fluid	0.19	0.23	1.23	-	-	0.04	0.92	0.46	1.28	2.73
Allantoic Fluid	0.44	0.61	6.06	-	-	-	23.31	1.64	1.08	-
Fetal Urine	-	0.07	0.45	1.20	ND	-	39.23	-	46.15	-

lysine was similar to that found in the respective tissues of the prolonged experiment (Table XII). The higher activity in this lysine infused fetus results from both the larger quantity of material infused (330 μ Ci compared with 250 μ CI in experiment C3) and the difference in time of sacrifice - immediate as opposed to 8 days. Maternal activity of liver and kidney medulla were of equal magnitude and unlike fetal tissues hepatic activity was less than that of the kidney cortex suggesting labelled material which has diffused across the placenta is as likely to be metabolised or excreted as incorporated into new maternal protein.

Amniotic and Allantoic Fluids and Fetal Urine

In the sheep allantoic fluid is present throughout pregnancy. As pregnancy progresses an increasing proportion of fetal urine is voided into the amniotic sac rather than via the urachus into the allantoic fluid[194]. This variability might be inferred from the activity in the amniotic and allantoic fluid and fetal urine (Table XIV , allantoic fluid having a higher activity than amniotic fluid in experiments C1, C2, C3, Ins C2 and Ins C3 but activity being similar in Ins C1. However a direct comparison between experiments is not possible without SA measurements, information on fluid volumes and the frequency of micturition. The lower activity in amniotic fluid may have resulted from a faster turnover of amino acids with between 300 and 600 ml water of a total volume of 1500 ml being exchanged within an hour in human pregnancy[194]. The majority of this is removed by fetal swallowing[31,32], and the low activity of amniotic fluid and increasing activity in fetal plasma in Ins C5, in which

infusion was directly into the amniotic sac, would support this. This rapid turnover would be beneficial if intra-amniotic nutritional supplementation of a fetus were to be practised.

As would be expected, little activity was found in the fetal urine obtained from experiments C2 and C3 in which the collection was made one week after the infusion. At this time free plasma amino acid activity was very low therefore even if concentrated by the kidney tubules little activity would appear in the urine. In the fetus of Exp C4 ^{3}H activity was surprisingly high. This may be related to the lability of the ^{3}H label (see p 220) and to the higher plasma water ^{3}H activity than ^{14}C activity in equivalent ^{14}C infusions. It may also result from the products of phenylalanine metabolism, with increased renal excretion of ^{3}H but retention of the carbon skeleton. The concentration of activity in Ins C2 and Ins C4 fetal urines, which were collected immediately the infusion finished, was 25 per cent and 34 per cent of plasma total activity and 67 and 100 per cent of the deproteinised plasma activity respectively. Without information on the frequency of micturition, the volume of urine produced, its dilution in amniotic and allantoic fluids and the SA in urine at a time of plateau SA in plasma, it is not possible to assess renal function and its influence on the composition of amniotic and allantoic fluids. In these experiments (Ins C2 and Ins C4) prior to sampling the SA of free amino acids in plasma had not returned to a plateau activity after the infusion of insulin. Therefore not only is the duration and volume of the

collection of fetal urine within the bladder unknown but also the SA of plasma during that time. However, the balance between fetal swallowing and urinary excretion[194] would suggest relatively large volumes of urine are formed, a result of lower water reabsorption capacity than in postnatal life[310]. The high activity of these fetal urines does not imply that there is an extensive loss to the fetus of these amino acids but rather that they may be recycled via gastro-intestinal absorption after swallowing.

CHAPTER IV

Discussion

This section is divided into two sections. In the first section the influence of animal management on amino acid metabolism, the metabolism of the infused amino acids phenylalanine, leucine and lysine, and the biochemical techniques for the measurement of amino acid and radioactivity are discussed. In the second section the data from the infusion experiments using ^{3}H, ^{14}C and ^{14}C amino acids with insulin is discussed with calculations of flux and plasma protein synthesis from the data compared with published data.

Section 1

a. Animal Management

Although acute experiments in which the uterus and/or its contents are manipulated under anaesthesia are now regarded as unphysiological, it is often accepted that normal physiological status exists in animals of chronic experiments. This assumption, without reference to any previously determined standard, can lead to difficulties in interpretation of data[2,3,298]. Pearson (1977)[2] examined chronic animal preparations similar to those used in the present studies and observed the length of time required to regain resting or pre-interference states in sheep after a variety of changes in the animals environment. The most important factors to be considered in adaptation to experimental conditions are as follows:-

1. Taming

Two forms of adaptation are necessary - the first to experiment rooms a new diet and management, and the second to being handled, repeated blood sampling and prolonged experimental procedures. Five to six weeks may elapse before the animal is "tamed" and adapted to its new environment. Plasma corticosteroid concentrations are elevated during the 4 - 7 days after removal from the natural field environment. Although the values then decrease, whether or not the sheep are handled, repeated venepuncture can be associated with an increase in the plasma cortisteroid level for as long as 36 days. Heart rate, initially raised, returns to pre-existing values by day7, but for 2 - 3 weeks can increase when the same attendant enters the pen. Plasma glucose, sodium and potassium concentrations and osmolality show no consistent changes.

2. Surgical Stress and Starvation Pre and Post Operatively

Surgery will alter the homeostasis of the animal. A 48 hour pre operative fast increases plasma cortisteroid values, and reduces glucose concentration. At operation plasma corticosteriods increase again and may remain elevated for 72 hours. Glucose and sodium concentrations remain relatively constant but potassium falls, increasing to normal again within 24 hours. Intravaginal temperature can fall by approximately 3°C during surgery, returning to normal within 6 - 6½ hours. For 2 days after operation feed intakes decrease by 10 - 50 per cent of the prefasting values, returning to normal in 3 - 4 days. Plasma glucose is raised above prefasting values for 7 days post operatively. The fetus requires a longer time (16 days) to recover from catheterisation stresses than the mother (7 days)[311,312].

Even minor stresses imposed on the ewe - for example a change in ambient temperature or pen cleaning can alter fetal breathing patterns, and changes in the level of nutrition, feeding pattern or water balance can cause a change in fetal fluid composition. 312, 313, 314.

Influence on Amino Acid Metabolism (Catabolism)

i. During Catheterisation and Recovery

Amino acid concentrations are influenced by nutritional status and operative procedures - fluctuations after catheterisation being present for 6 - 12 days[315]. Young et al 1975[64] studying effects of cannulating the fetal jugular vein found many changes in fetal plasma. The total free amino acid concentrations increased by 13 per cent during operation, with an increase to 40 per cent above the normal taurine concentration and 44 per cent above the usual amino isobutyric (AAB) acid concentration. Basic amino acid values, however, decreased by 10 - 30 per cent 24 hours post surgery the AAB value was still increasing, as were also 1- and 3- methyl histidine (IMH and 3MH) values. The concentration of glutamic acid was reduced by 30 per cent lysine by 18 per cent, alanine by 32 per cent and the branched chain amino acids by between 9 and 32 per cent. In the period from the 3rd day until the end of the second week glycine values returned to normal and then declined. The BCAA, after their initial reduction, increased - isoleucine to 23 per cent, leucine to 36 per cent and valine to 42 per cent above normal values and thereafter remained high. Taurine AAB, IMH and 3MH remained approximately twice normal, but basic amino acids were at control

levels. Infusion of aminosol glucose into the fetus had no effect on the blood amino acid patterns.

Clearly, any attempt to assess normal fetal total or individual free amino acid concentrations during this period could lead to very misleading results. The decrease in glucogenic and increase in branched chain amino acids similar to the pattern observed in starvation suggests placental transport may be impaired. However, apart from maintaining blood urea concentrations (in controls urea decreased), aminosol glucose had no effect on the pattern of amino acids. The increases in the taurine and methyl histidine values suggest possible liver and muscle catabolism and possible inhibition of the citrulline-arginine cycle is suggested by the observed high citrulline and low arginine concentrations. Any assessment of the rate of turnover of metabolic functions until homeostasis is regained could therefore lead to erroneous conclusions.

iii. In Fed and Fasted State

Changes of free amino acid values in the fed and fasted state were studied by Cross et al 1975[316]. An overall reduction of essential amino acids, and an increase in glycine were found in the fasted state, which also caused an increase in total amino acid half life, 6.2 $\pm$ 0.98 minutes compared with 5.7 $\pm$ 0.51 minutes in the fed group. Short term changes in the peripheral blood during feeding, although present, are not normally large because of the protective action of the liver. Feeding can however produce other effects - such as increased oxygen consumption

heart rate and blood pCO_2 and a fall in blood pH and plasma volume. To minimise these changes pelleted food was offered hourly. Plasma insulin and growth hormone secretion fluctuations were also stabilised by this practice. Fetal fluids, including plasma and urine, show a marked alteration in glucose and fructose concentrations with changes in feed intakes. Feed was offered to maintain blood glucose concentrations between 3.00 and 3.50 mmol/l - that being the concentration normally found under field conditions.

3. Circulatory Changes and Blood Distribution

Circulation in the fetus near term is fast - one "circuit" being complete in 12.6 seconds ($\pm$ 1.0 S.E.M.) [307], with [306] 33 to 42.5 per cent[307] of the flow being directed through the ductus venosus. This rapid transfer can result in any changes in the placenta or peripheral tissues quickly affecting one another. Previously Power et al (1967)[317] had found that a large proportion of the placenta of dogs and sheep do not normally receive blood in optimum quantities to provide efficient oxygen exchange. Labelled aggregates of albumin were unevenly distributed among and within cotyledons but if hypoxia occurred a more uniform distributions increased flow and improved the efficiency of exchange. Cohn et al (1974)[318] studying hypoxemia and acidaemia in 122 - 142 day fetal lambs showed that although blood flow to the total body decreased with increased proportions being directed to the heart, brain and adrenals, umbilical vessel blood flow was maintained with an increase of 41 - 57 per cent of the

flow being directed to the placenta. Transfer "facilities" on the fetal placenta side therefore appear to be well protected. However, Duncan and Lewis (1969)[319] found a decrease from 60 per cent to 32 per cent of the uterine blood flow was directed through the maternal placenta during maternal hypoxia in rabbits. In the human, hypoxia raised maternal blood glucose levels but fetal blood glucose levels either remained constant or decreased. If the dissociation was associated with an oxygen dependant carrier mechanism the discrepancy would be explained[320]. Hypoxia and acidaemia are therefore both important considerations in animal preparations because of their possible direct influence on transport mechanism and more indirect effect on blood flow and distribution.

It is worth noting heart rate increased for 2 - 3 weeks when the pen of "untamed" sheep was entered[298] and this could affect observations on the placental transfer of metabolites.

A placental structure to allow maximum exchange is important. To interpret affects of haemorrhage or ligation knowledge of placental structure is required. In human placenta the typical pattern of one single villous stem and one maternal ostium supplying each lobule has been shown to be incorrect[321]. Dissection and graphic reconstruction indicate that one villous stem supplied several lobules, which were formed from several stems and arterial ostia. These formed a network at the base of the placenta and determine the distribution of lobules

Blood Volume and Haemodilution

Fetal haemorrhage in sheep was found to be associated with a rise in fetal plasma ACTH and arginine vasopressin but no

consistent trend in hormone growth[322]. Some of these findings could however be associated with exteriorising the fetus. Changes occurred after a 20 per cent loss of blood volume and younger fetuses were more sensitive to this with a greater fall in blood pressure and heart rate than older fetuses. These findings are similar to those changes occurring under hypoxic conditions and surgical stress and may well affect fetal blood flow in a similar manner. Removal of blood for sampling would be equivalent to haemorrhage loss. The volume of blood taken (10 mls per sample) from the ewe would have little effect on the total blood volume of the ewe - approximately 3.5 litres. The fetus, however, only has a total blood volume of approximately 200 - 300 mls. This limits both the size and frequency of sampling. In most experiments approximately 2.5 ml/hour were taken

4. Circadian Rhythms

Actual clock times are noted in the animal methods section. Plasma amino acid concentrations have been shown to be maximal between 12.00 and 20.00 hours and minimal at 04.00 in adult[323] man and also the neonate only 48 - 72 hours old[324]. The sheep were penned in a room with constant artificial light to minimise changes and the majority of experiments were carried out during the day.

Experimental Conditions of Infusion Studies

In present experiments, the ewes had been kept penned indoors and handled frequently for several weeks before implanting the catheters. These could not be inserted before day 80 because of

fetal tissue fragility, at this time placental growth being complete and rapid fetal growth being about to commence. Because of the limitations imposed by time of insertion of catheters and recovery from this procedure only the latter part of pregnancy can be studied using this technique. At least 11 days (with the exception of the first tritium infusion) were allowed for recovery following surgical catheterisation, when amino acids and metabolism changes would be returning towards the norm. As amino acids may be utilized as a secondary metabolic fuel it was important that additional stress demands had been abolished. An increased utilization of the amino acids would not necessarily have been reflected by a decrease in plasma levels, but by a decrease in the half lives and this would not have been readily detected unless compared with controls of longer recovery periods. This increase in usage could be met either by increased catabolism or by increased transfer of amino acids from mother to fetus. Any blood circulatory changes either on the maternal or the fetal side of the placenta could also alter the transfer rates of amino acids.

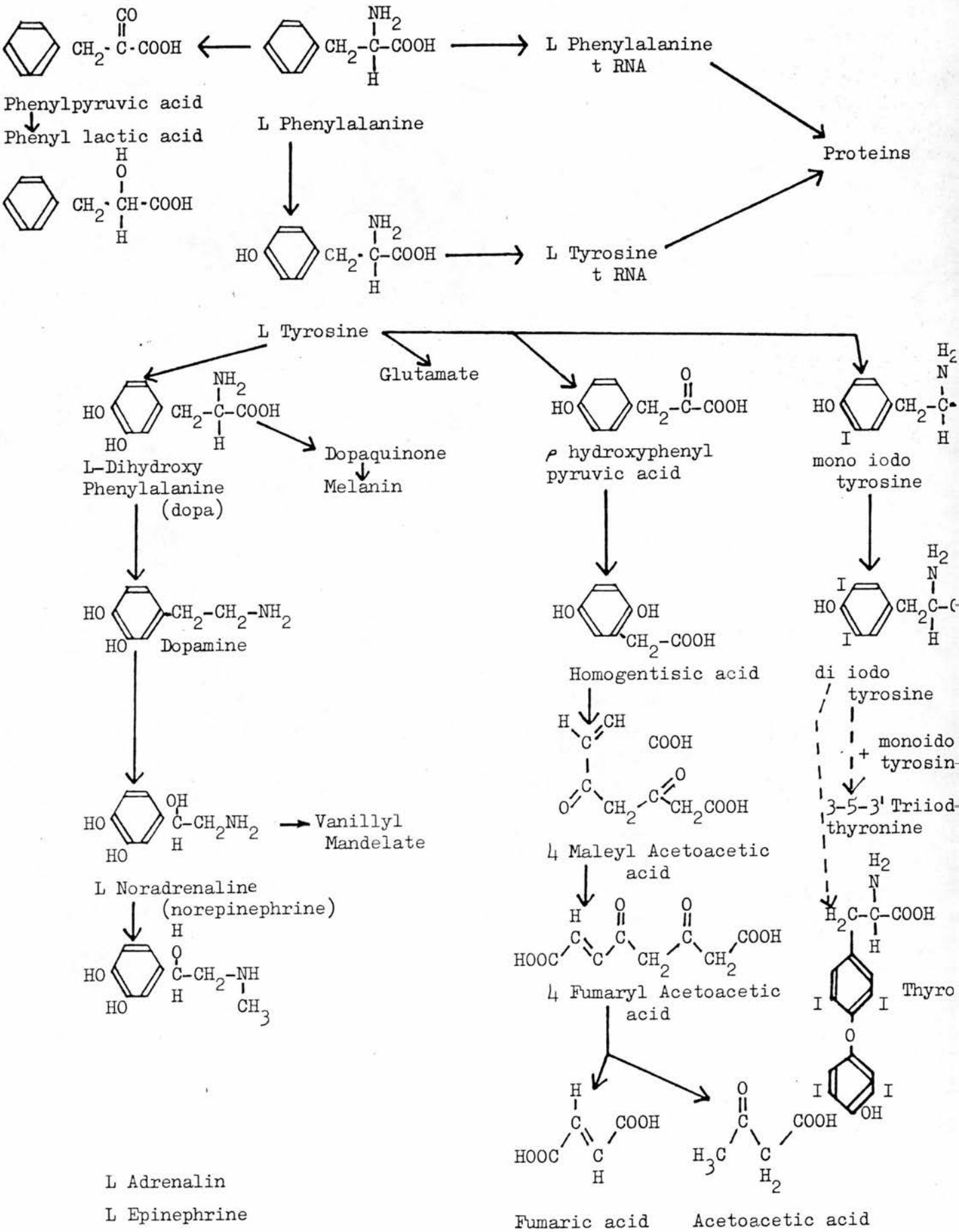
Conclusion

The sheep used in the experiment were prepared for experiment to produce a minimally stressed animal. This was achieved by application of the following procedures:-

1. The sheep was "tamed" and used to handling and prolonged experimental procedures involving repeated blood sampling.
2. Adaptation to environment, feeding regimes and daily management was complete. Pens prevented large energy expenditure on movement.

Fig 1

L Phenylalanine Metabolism



3. Hourly feeding minimised changes occurring with feeding and absorption mechanisms.
4. Recovery time from catheterisation stresses was allowed.
5. Frequency and volume of fetal blood samples were restricted.

b. Biochemical Analysis

1. Phenylalanine, Leucine and Lysine Metabolism

Three L amino acids - phenylalanine, leucine and lysine were infused into sheep. The subsequent metabolism of the individual amino acids are as outlined in Figs 15,16 and 17 The products of metabolism of the universally labelled ^{14}C amino acids will contribute towards the plasma water total activity, although the contribution would be small in comparison with the parent compound - tyrosine activity and SA being small in comparison with that of phenylalanine. The ^{3}H label of phenylalanine appeared to be more labile and ^{3}H metabolites of phenylalanine accounted for a larger percentage of plasma water activity, (See page 249) than the ^{14}C isotope of phenylalanine.

Phenylalanine

Phenylalanine metabolism is complex, in that it is the precursor of several important metabolic compounds including noradrenaline, adrenaline, (norepinephrine, epinephrine), thyroxine and melanin. If not required for protein synthesis the first step in phenylalanine metabolism is hydroxylation to tyrosine, catalysed by phenylalanine hydroxylase - the enzyme

deficient in most forms of phenylketonuria. If phenylalanine hydroxylase is deficient transamination to phenylpyruvic acid, requiring pyridoxal phosphate as co-factor is increased. Tyrosine is transaminated to p-hydroxyphenyl pyruvic acid by the enzyme tyrosine-glutamate aminotransferase. The synthesis of this enzyme in the liver is increased by increased tyrosine concentrations, adrenal steroids, insulin and glucagon but is decreased by hypophyseal somatotropin. The degradative pathway of tyrosine is via p-hydroxyphenyl pyruvic acid (P.H.P.P.) which is subsequently converted to homogentisic acid. The copper containing enzyme P.H.P.P. ase activity is low in fetal liver and this accounts for the relatively large urinary excretion in preterm infants of phenyl lactate metabolites. Lack of ascorbic acid, a cofactor, can also increase tyrosine concentration. Further metabolism of homogentisic acid to 4-acetoacetic acid and 4-fumaryl acetoacetate requires a ferrous enzyme, molecular oxygen and a high concentration of reduced sulphydryl compounds, e.g. reduced glutathione.

The C atoms of the phenylalanine molecule appear in the final metabolite, acetoacetate, as follows:-

α C becomes the carboxyl C atom of acetoacetate

C 2 of the ring is the precursor of the carbonyl carbon

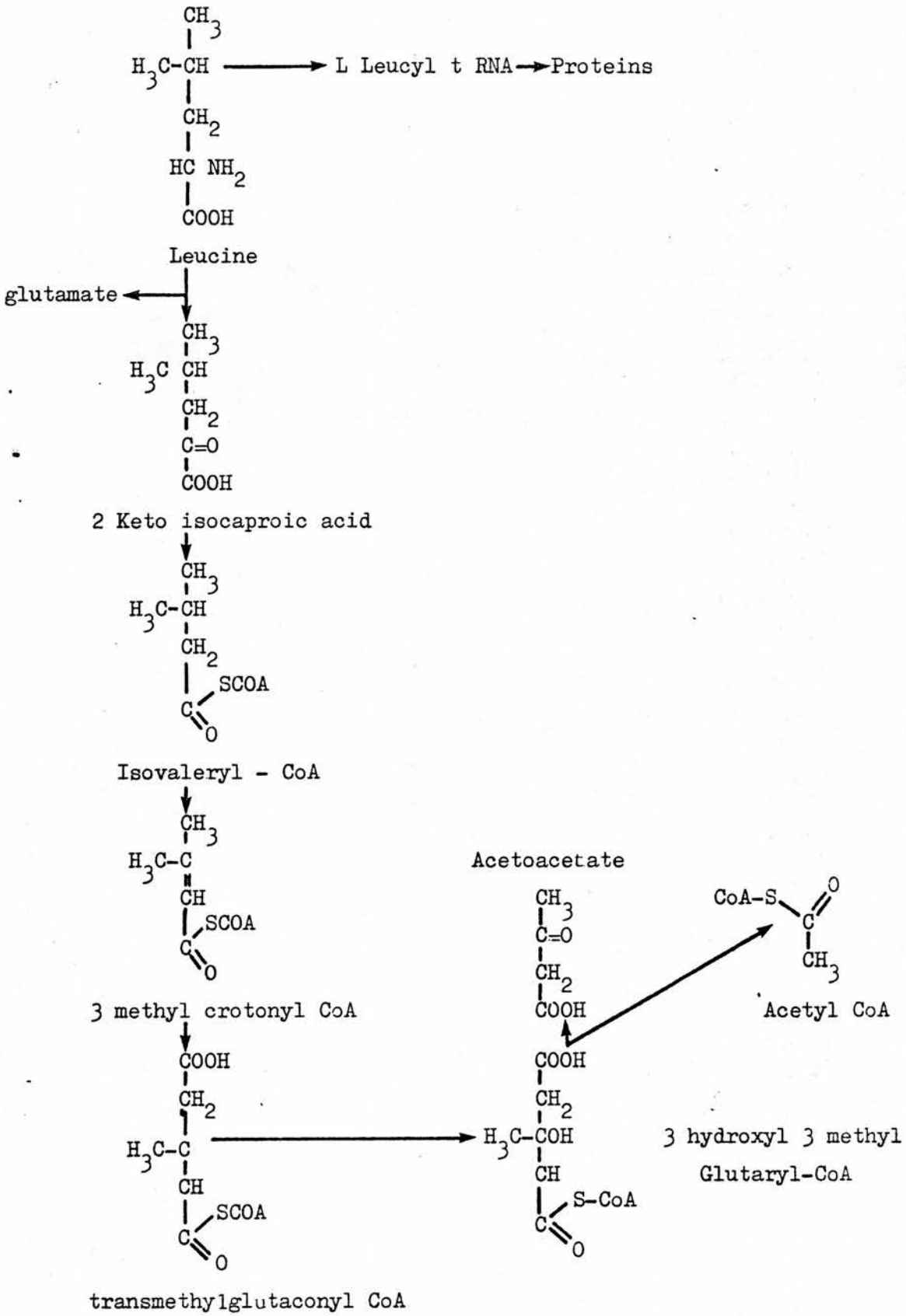
C 1 or 3 of the ring is the precursor of the terminal carbon

and

β C becomes the C of acetoacetate.

The other C atoms of the ring in position 1 or 3, 4, 5 and 6 are present in the fumarate molecule.

Fig 17 L Leucine Metabolism



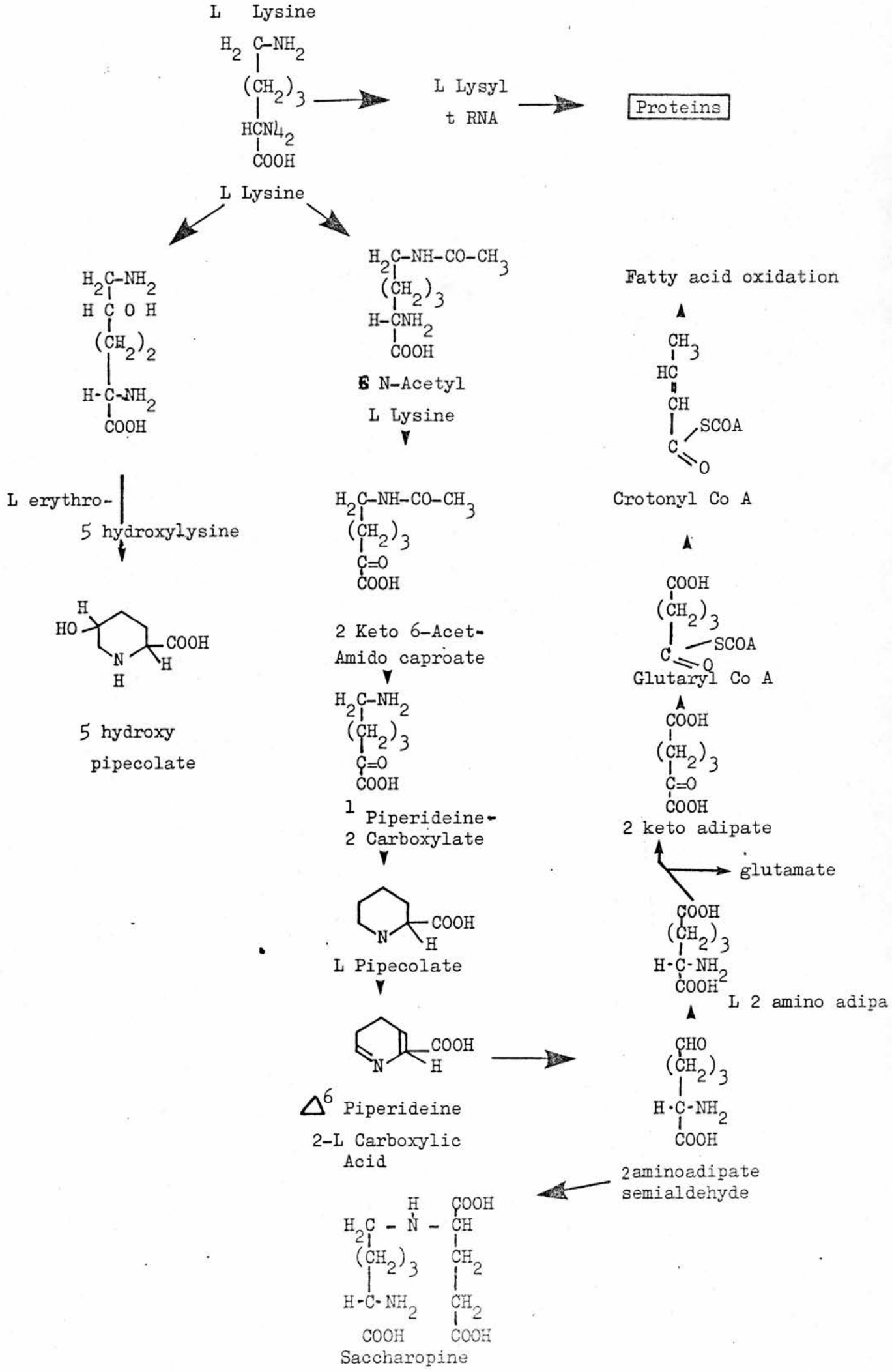
Two important synthetic pathways with tyrosine as precursor exist. The first, catalysed by tyrosinase, a copper containing enzyme is through DOPA and dopamine to adrenaline, AMP, GMP, UMP, CMP, pyridoxal phosphate and ascorbate being cofactors. Alternatively, DOPA may be diverted via a leucocompound to melanin. Secondly, iodination of tyrosine requires a copper containing enzyme for the production of thyroxine.

Leucine

Leucine degradation shares a common catabolic pathway with isoleucine and valine - i.e. (with the exception of a leucine specific aminotransferase present in normal liver) enzymes for the branched chain amino acids are non specific. Branched chain amino acids are deaminated to their α- Keto acids before oxidative decarboxylation to the acyl Co A derivative - which has one less carbon atom than the parent amino acid. Biotin is required for conversion of 3-methyl crotonyl Co A to 3-methyl glutaryle Co A and final degradation is through fatty acid oxidation.

Leucine is the only amino acid known to stimulate protein synthesis and inhibit proteolysis (see Chapter 1 section 6). Additionally, a large proportion of leucine metabolism occurs in muscle, the tissue most sensitive to altered energy availability, and plasma branched chain amino acids show a greater reduction in concentrations than any other group of amino acids in starvation (p 71). This suggests that leucine may have an important role in balancing protein synthesis and catabolism. Reduced energy availability also increased branched chain amino acid oxidation in muscle, possibly depleting a critical compartment within the

Fig 1 Lysine Metabolism



cell and thereby turning protein metabolism towards proteolysis rather than synthesis.

Relatively little leucine is extracted by the liver therefore the concentration of labelled leucine in peripheral blood may be less affected by immediate hepatic uptake of infused leucine on the first passage through the liver than the amino acids phenylalanine and lysine. Incorporation into hepatic proteins, including those subsequently released into plasma may thus appear to be lower, as it is possible that the intracellular phenylalanine and lysine specific activities in the hepatocytes are higher than would be suggested by the corresponding plasma specific activities - intracellular hepatic activity being reported as 40 - 60 per cent of that of the plasma specific activity. [325,326]

Lysine

Metabolism of lysine is restricted to protein synthesis and lysine does not participate in transamination reactions. This reduces recycling and thus frequently makes lysine the amino acid of choice for studies of protein synthesis and catabolism. Cofactors required for the degradation of lysine are NAD (P), NAD (P)H, FAD and magnesium. Final catabolism, like leucine, is through fatty acid oxidation of crotonyl CoA.

2A. Amino Acid Analysis

Moore and Stein (1951)[327] originally developed the ion exchange chromatograph method of amino acid analysis. Methods for microdeterminations were then developed[328] and improved resolution of the amino acids were achieved with lithium buffers[329]. An adaptation of this method[297] (Cockburn 1971) was employed in the present experiments. Preliminary preparation of biological fluids for amino acid analysis involves a deproteinisation step.

Picric acid was first used, but as this interfered with colour production in the ninhydrin reaction excess had to be removed by passing through a Dowex column. Comparison of the free amino acid concentrations of plasma deproteinised with picric acid and sulphosalicylic acid (SSA), which gave no interference, gave similar results for both - the coefficient of variation for individual amino acids of both methods being 0.0 - 6.0 per cent [27,330]. Phenylalanine recoveries were between 92.7 and 108.8 per cent and leucine and lysine between 82.7 and 116.9 per cent. Stein calculated that 95 - 100 per cent of the total and α amino nitrogen in plasma could be accounted for by the sum of the individual amino acid values. Traces of protein in the sulphosalicyclic acid supernatants from samples precipitated with 0.5 ml 3 per cent SSA to 0.1 ml plasma were reported by Dickinson,[27] but these were not eluted from the column during a blank run. Perry et al 1968 used 30 mg solid SSA: 1 ml plasma[329], Hamilton[328] using 3 per cent SSA, having found no significant difference between this and picric acids. Aoki et al 1973 found no difference between 10 per cent and 20 per cent SSA or 6, 10 or 30 per cent perchloric acid for whole blood

The 0.6 mol/l SSA used throughout these analyses is equivalent to 15.25 g/100 ml.

Tissue and plasma samples were stored at a temperature of -70°C at which temperature amino acid concentrations, with the exception of glutamine and glutamic acids are stable[27].

ii. Tracer Infusion Techniques

Three types of isotope dilution methods have been employed - single injection, continuous infusion and primed continuous infusion. The mathematical treatment and theory of these tracer techniques and compartmental analysis are discussed by Shipley and Clark[332]. White et al 1969 evaluated and mathematically compared these three methods using ^{14}C glucose[333]. The same "irreversible loss" i.e. flux or entry rate of glucose was found for all three methods but the pool size and space appeared higher in the primed infusion than the single injection. Total entry rate can only be calculated from the latter. Daily feeding compared with 12 feeds given hourly gave essentially the same results, but the first regime had a greater variance. Using glucose the ratio of bolus quantity to infusion rate was important, ratios P (priming injection in nCi): F (infusion rate in nCi/min) between 69:1 and 98:1 leading to more consistent results. For any of the results to be valid all glucose (or tracer used) entering into the system must mix in the sample pool under relatively steady state conditions and there must be no re-cycling. An indication of recycling may be a slow equilibrium, although slow turnover can also produce this effect. Amino acid tracer infusion techniques have been employed to calculate

protein turnover and synthesis rates, [326,334,335,336,337,338,339,340] based on that of Waterlow and Stephen [164,327,341].

Equilibrium (plateau) specific activity is reached in a shorter time in a primed infusion than a continuous infusion alone, and the effect of insulin on this plateau can be observed (single injection experiments would not allow this). For these reasons the primed infusion technique, with a P:F ratio of 60.3:1 was used in these experiments and the sheep were fed every hour.

3. Radioactivity Measurements

i. Tritium

Although tritiated amino acids have been used both in in vivo and in vitro studies, the differing stabilities of the ^{14}C and ^{3}H isotopes are seldom considered in discussions. Hider et al (1969)[157] found the ^{3}H amino acid was not stable and lost radioactivity on incorporation into protein. Evans et al (1963)[309] incubating DL amino acid with a general tritium label (G ^{3}H) in the presence of renal D amino acid oxidase found the specific activity of the L compound to be lower than expected (i.e. the same as the parent DL compound). The percentage loss was equivalent to the percentage of the label found in the hydrogen attached to the (2) carbon atom - alanine (G) 57 per cent (* see foot note) leucine (G) 44 per cent, leucine (2T) 17 per cent, phenylalanine (G) - 32 per cent and tyrosine (G) 39 per cent. Konikova et al in 1947[343] had previously noted glutamic - alanine "aminopherase" (transferase) resulted in a labile α hydrogen molecule - even after heating the experimental enzyme system.

Waterfield 1968[343] studying tritium exchange in dilute aqueous solution also found some lability - L-Dopa 256T -

* Foot note number indicates position of hydrogen atom replaced by tritium T - G indicates general labelling.

15 per cent, L-leucine 4 - 5T - 1 per cent, L-phenylalanine 4T- 2 per cent L tyrosine, (side chain 23T) - 1 per cent and L tyrosine 3 - 5T - 8 per cent.

A similar reaction to that of D-amino acid oxidase is suggested by Hider[150] for enzyme involved in the activation of amino acids for protein synthesis. Examining L (G ^{3}H) leucine, with 50 per cent label and glycine with 100 per cent label on the α hydrogen atom, the glycine appeared more labile, proportionately less ^{3}H being bound when incorporated into protein than with leucine. Using ^{14}C and ^{3}H labelled amino acids and correlating the specific activity of the 2-labelled forms in the medium and the corresponding ^{14}C ratio incorporated after 30 minutes, Hider calculated correction factors of 22 per cent for ^{3}H leucine and 37 per cent for ^{3}H glycine.

The use of tritium labelled compounds can thus give misleading results, particularly if employed in protein synthesis studies or dual isotope experiments. For this reason the experiment of Year 1 using tritiated phenylalanine were repeated in Year II with ^{14}C labelled amino acid.

In addition to the problem of tritium lability, chemiluminescence is encountered. This is particularly marked in whole blood samples. Chemiluminescence is produced by spurious events generating a single photon, still detected by coincidence liquid scintillation system because of the number of random events. This gives rise to a high background countrate and because this varies from sample to sample, its effect cannot be eliminated. The problem of chemiluminescence is very significant when counting samples of low activity. It can be diminished by neutralising the sample

(alkaline proteinaceous material is usually associated with chemiluminescence) or awaiting the decay which is temperature dependent, of the spurious activity.

ii. Total and Specific Activity

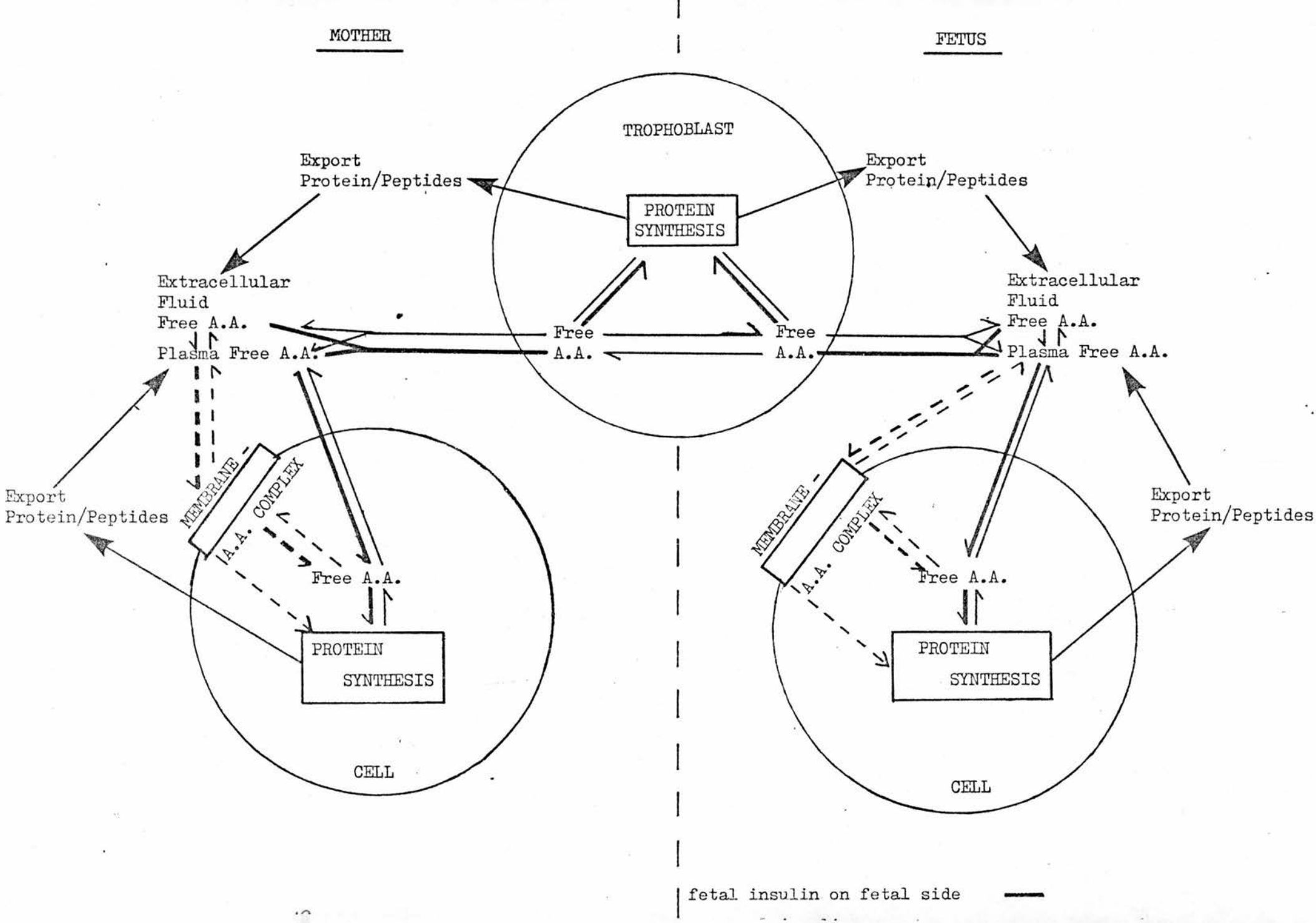
The most frequently determined and useful measurement of radioactivity is specific activity SA i.e. the quantity of radioactivity (disintegrations per minute, d/min), per given quantity of the compound - amino acids in these experiments. However, the methods used to obtain specific activity measurements are technically difficult and require a level of activity sufficient to enable accurate measurement of activity in the relevant fraction from ion exchange chromatography. For these reasons, in addition to SA, blood plasma and plasma water total activities have been examined, allowing comparison between experiments and showing simply label movement. Whole blood and plasma radioactivity measurements include both free and bound labelled amino acids and its metabolites, but plasma water (i.e. deproteinised plasma) measurements reflect only the free amino acid, soluble peptides or amino acid metabolites. A rapid and continuing incorporation of the amino acid into protein, as opposed to a steady, relatively low concentration of activity in plasma water has the effect that only whole blood and plasma samples obtained soon after the commencement of label infusion would be of appreciably mixed origins, the activity of later samples being principally that of protein bound or protein incorporated amino acids. Fetal deproteinised samples may contain small peptides, the centrifugation procedure employed leaving a slightly opalescent supernatant, but

these peptides would not be expected to elute with the free amino acids on column chromatography.[27]

In the results and discussion sections reference to activity or radioactivity refers to the measurement of this total activity and includes all species of labelled molecule and not only that of the infused species. Specific activity refers to measurement of the radioactivity present in a particular compound (amino acid). A plateau measurement of radioactivity in a system can only be achieved when the amount of radioactivity entering that system is equal to that leaving the system. This implies that the system must first be saturated so that continued accumulation of activity does not occur.

Specific radioactivity measurements eliminate changes in these measurements due to dilution or concentration of the pool of the compound (in these experiments individual amino acids) and at equilibrium will remain constant for a given pool size. Also metabolism of the amino acid to other labelled compounds will not interfere with SA measurements or calculations of e.g. flux from these SA measurements. Any change in SA occurring once equilibrium has been achieved represents a change in flux into or out of the pool, and such change may be detected even although the concentration of the compound may remain constant.

Development of a plateau of SA or radioactivity during constant infusion of a labelled material will occur when the infusion rate of labelled material is equal to the rate at which labelled material leaves the given pool. Injection of a bolus of radioactivity will speed the development of this plateau during infusion experiments by decreasing the time


MOTHER
FETUS
TROPHOBLAST
Export
Protein/Peptides
Export
Protein/Peptides
PROTEIN
SYNTHESIS
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fetal insulin on fetal side

until saturation point is reached. Provided that the concentration of infused material is very small in comparison with the total pool size it enters the infused material will mix with the unlabelled material in increasing amounts until a maximum is reached. This maximum plateau is reached when a constant proportion of material is labelled. The infused labelled material then leaves the pool with unlabelled material in the same proportions and at the same rate as it is entering. At this point the flux may be calculated from the plateau SA measurements. The length of time to reach equilibrium and the proportion of labelled to unlabelled material will depend on the size of the pool, (i.e. the amount to be saturated) the quantity and rate at which the labelled material is infused and the flux into and out of the pool. At equilibrium the pool size and the flux will be constant so the proportion of labelled material i.e. SA will depend on the infusion rate. At high flux and slow infusion rates there would be a long lag phase before a plateau of activity could be reached - and it might never be achieved. A fast infusion and slow flux would conversely decrease the period of time until the plateau is reached, but too fast a rate might result in mixing and concentration changes which would obscure slow changes in flux. In these experiments labelled radioactive amino acids were infused into the blood circulation. The situation is represented in Fig 19 .

There is a net positive flux of amino acids from mother to fetus with the result that labelled amino acid concentrations in the fetus would be expected to increase faster and to a greater extent during maternal infusions than the maternal specific activity would increase during fetal infusions.

For plateau specific activities to be reached all transport functions within the system must be constant. During the experiments maternal and fetal blood were the only readily measurable compartments of the system and tissue cells remain as a "hidden" compartment. At termination, the radioactivity in tissue cells can be determined but it is unlikely that equilibrium with the possible exception of free intracellular amino acid pool would have been reached in such short term experiments. Tissue activities could only be confirmed by serial biopsy or timed termination experiments. In longer term experiments equilibrium between tissues should have been achieved, but this cannot be known with certainty. Additionally, it should be noted that rapid exchange between plasma and extracellular fluid compartments has been demonstrated (Chapter 1 section 4). Discrete compartments, although utilized for some mathematical calculations, may not therefore exist.

c Calculations

b. Incorporation of Amino Acids into Protein

The rate of incorporation of amino acids into protein is calculated from the ratio (S_B/S_i) of the S.A. of bound amino acid (S_B) to the S.A. of the precursor amino acid (Si) at equilibrium. Protein half lives and turn-over may be calculated from the incorporation rates (See Appendix 12)

In these experiments incorporation rates of amino acid into tissue protein have not been measured. Tissue was taken from experiments C1 to C4 but in these experiments sacrifice was at 7 - 9 days past infusion. Extensive recycling could therefore have occurred and very little of the total activity would be expected to be present in the plasma water. Total activity measurements were made in these tissues (Chapter 3 section 2) a major proportion of this activity being accounted for by the bound labelled amino acid in protein - the ^{14}C phenylalanine bound activity which was found to be present in the hydrolysates from C1 and C4 accounting for over 90 per cent of the total activity in plasma protein. In the series of experiments Ins C1 and Ins C5 the three hour period of insulin infusion 2 - 3 hours prior to sacrifice could have caused a considerable alteration in the S_B/S_i ratios. Infusion of insulin at 0.26 - 1.66 iu insulin/kg/hr in fetal lambs of 135 days gestation reduced plasma glucose and the intracellular free amino acid content of heart (70 per cent) liver (40 per cent) and muscle (90 per cent) but not of brain[340]. The S.A. of the intracellular leucine of heart, liver and muscle also increased. Although insulin infusion rates in these experiments were slightly lower (0.18 - 0.31 iu/kg/hr) to

those of Chrystie et al (0.4 iu/kg/hr)[333] plasma insulin concentrations were similar in the fetal lambs of these experiments Ins C1 - Ins C5, including the fetus of Ins C3 in which there was no change in plasma glucose. As in the experiments of Chrystie et al in which there was no change in S_b, total label uptake rate showed no alteration on starting and stopping insulin, although the SA of plasma and therefore probably S_i increased. Equilibrium may not therefore have been re-established after the 3 hour insulin infusion. In rats equilibrium i.e. plateau SA only occurred in serum and liver after three hours of continuous lysine infusion and in muscle after 6 hours[326], tissue SA's were not therefore measured, the acceleration of plateau SA by use of a primed injection probably having little effect after altering the metabolic equilibrium by infusing insulin.

In all calculations of protein turnover and synthesis rates the SA of the precursor amino acid has to be determined. Most calculations assume intracellular free amino acids to be the precursors but several studies have indicated that extracellular or combined extracellular and intracellular amino acids could be the precursor - possibly an amino-acid-membrane complex being the actual precursor for protein synthesis (Chapter 1 Section 4). Changes in the source of precursor or differences between SA of intracellular and extracellular amino acids could significantly alter the apparent synthesis rates. This could be particularly important if insulin were to alter the equilibrium

between intracellular and extracellular amino acids. The maximum SA would not however be greater than that of plasma.

An estimate of amino acid incorporation into mixed proteins released into fetal plasma was made from the data. The assumptions made in the calculations, with the drawbacks of these are as follows:

1. The SA of plasma amino acids at the end of the measured period of time (usually an hour) was assumed to be the precursor SA. As most plasma proteins are synthesised in the liver the SA of the precursor for these should be used. This is normally assumed to be that of the intracellular amino acid - approximately 60 per cent that of plasma (Waterlow)[326] although there is evidence that extracellular amino acids may be utilized. Rapid changes caused by insulin infusions would have resulted in a changing SA during the time intervals. A higher assumed Si than actually present would reduce the apparent synthesis rate.

2. The SA of the bound activity was based on the acid precipitated fraction of plasma (i.e. total activity - the free amino acid activity). This would increase the apparent activity as activity from products of metabolism and transamination reactions would be included. However activity from these sources would not be expected to be large. The activity of plasma water was not used as the presence of small peptides or degradation products in the deproteinized plasma was not excluded. Thus apparent bound SA i.e. μmol/g protein would also be high - but this would increase the apparent synthesis rate. Total

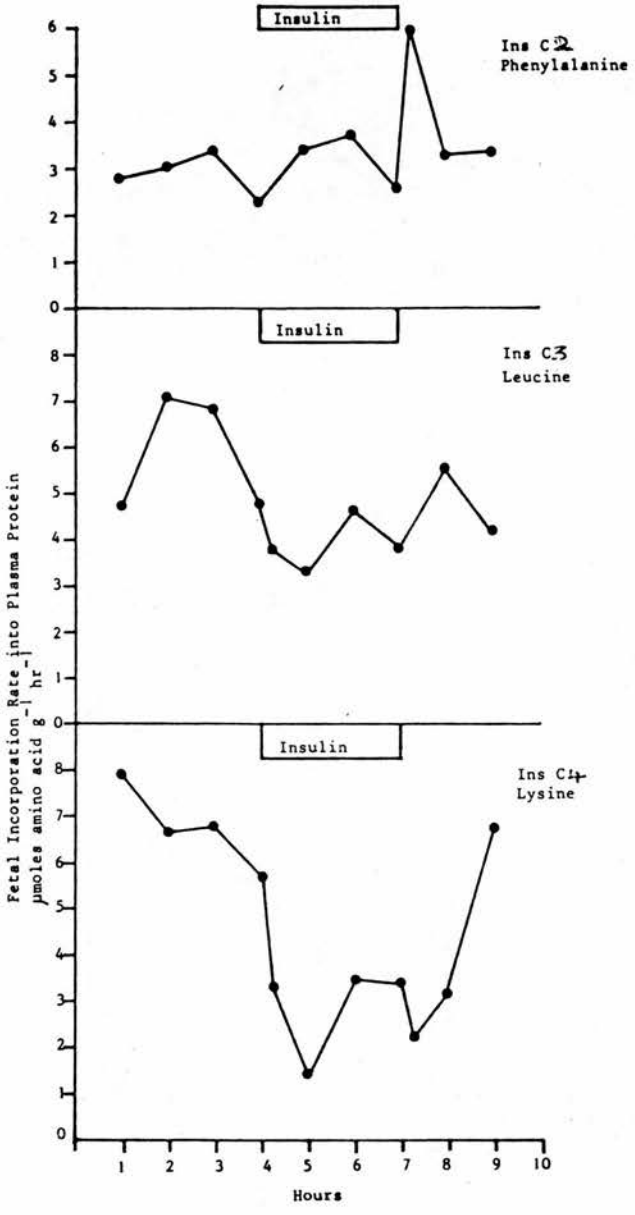


Fig 20 :

Incorporation rate of amino acid into plasma protein

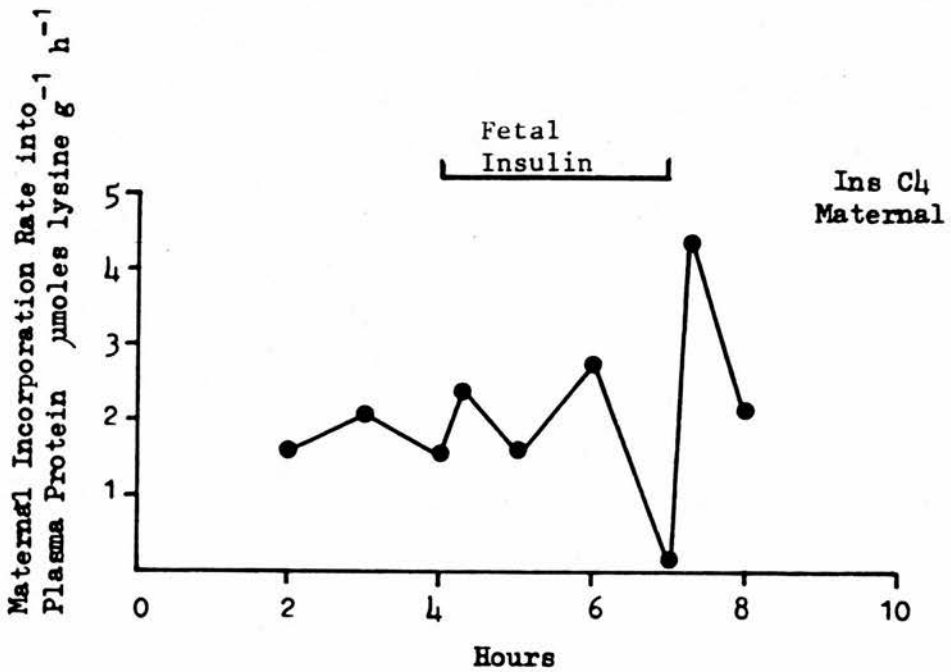


Fig 20ii

Incorporation rate of amino acid into plasma protein

plasma protein was measured (Fig 20 and Appendix 10). However, only newly synthesised protein released into the plasma would be detected, thus synthesis rates would depend not only on synthesis but on rates of release into and removal from the plasma.

In both Ins C3 and Ins C4 (and Ins C2 at 4 hours) the lowest rate of amino acid incorporation was recorded during insulin infusion. However, in Ins C3 and Ins C4 infusions the rate was falling prior to insulin infusion. In Ins C3 and Ins C4 a rapid increase in synthesis (or release) occurred within 15 minutes of stopping insulin, but in Ins C4 the increase to pre-insulin values only occurred after 2 hours, decreased "incorporation" being observed at 15 minutes post insulin. Plasma free lysine SA increased at this time, possibly indicating a brief reduction in net synthesis rate when insulin was withdrawn. Unlike SA measurements, leucine incorporation showed the greatest change on stopping insulin, incorporation increasing from 3.83 to 11.44 μmols leucine per gram of protein per hour in 15 minutes.

From Sephadex separation of plasma protein (part (2) page 235) the major portion of activity was associated with the albumin fraction. Additionally a larger proportion of the phenylalanine is present in a small peptide precursor (Chapter 1 Section 4). A more rapid response in SA of the albumin precursor may also occur than in albumin itself.

Maternal lysine incorporation rates were lower (mean 2.05 $\pm$ 1.18 μmol/g/hr) than the equivalent fetal rates (4.62 $\pm$ 2.21 μmol/g/hr) and showed little change until after

3 hours of fetal insulin infusion, when only 0.09 μmoles lysine were incorporated into protein in an hour. This could reflect preferential catabolism of albumin (suggested to be a storage form of amino acids [162]) to meet the increased fetal demands of this period. As these reduced on stopping insulin and maternal amino acids were again available for synthesis, a surge of albumin - (total protein increased from 56.8 g/l to 61.9 g/l) may be released into the plasma to replace that utilized for fetal supply - resulting in the incorporation rate increase from 0.09 to 4.38 μmol lysine per gram tissue per hour.

Overall incorporation rates for both maternal and fetal infusions are probably low - as the Si value of precursor activity based on plasma is liable to be more accurate than that of SB, the protein bound SA. In vitro determination of the rate of leucine incorporation into tissue albumin (of adult man) [188] showed that leucine was incorporated at 1.0 ± 0.1 μmol/g/hr if incubated in medium with amino acids at plasma concentrations (1.2 ± 0.1 μmol/g/hr if insulin were present) but to be 3.1 ± 0.4 μmol/g/hr if incubated in medium with amino acid concentrations 4 times that of the plasma. Fetal turnover rates have been shown to be greater than those of adult tissues [336, 339, 340] the higher intracellular plasma free amino acid concentrations of the fetal cell being a likely contributing factor. However, incorporation rate of leucine into hepatic albumin (incubation at 4 times plasma 4 hr) was 5.1 ± 0.6 μmol/g/hr but only 0.87 ± 0.17 μmol/g/hr in the albumin released into the medium.

TABLE XV

C. Activity in fraction of Sephadex column chromatography eluate

Experiment	Sample	Time	Activity d X 10^3/min/ml plasma			Fraction 4 / Fraction 2
			Fraction 2	Fraction 4	Total Plasma	
C1	Maternal	3½ hour	0.67	5.66	6.50	8.45
		8 days	1.31	11.12	13.25	8.49
	Fetal	4 hour	0.85	4.10	8.40	4.82
		2 days	1.20	4.32	9.50	3.60
C2	Fetal	2 days	10.32	39.62	85.00	3.84

2. Sephadex separation of Plasma Protein

Sephadex G200 was used to separate plasma proteins into fractions of similar molecular weight. The presence of a protein fraction in the eluate was detected by monitoring absorption at 280nm.

Plasma from maternal samples taken at 3½ hours and 8 days (Experiment C1) and fetal samples taken at 4 hours (Experiment C1) and 2 days (Experiment C1 and C3) were applied to the Sephadex column. The 280nm absorption trace of the eluate from Sephadex columns after applying either maternal or fetal plasma is shown in Figure S1. Six fractions were discernable from maternal plasma but only two from fetal plasma. The probable composition of these fractions is indicated on the figure.

From figure S2 and table XV, which gives the radioactivity associated with each plasma protein fraction, it can be seen that over 78 per cent of the activity was present in the albumin fraction in the fetus and over 89 per cent in that of the mother, the remaining activity being associated with a mixed α and β globulin fraction. No activity was detected in the maternal plasma γ globulin fraction. Baseline activity of the column eluate during the period of α and β globulin elution and activity associated with fraction 2 increased with time. However, in maternal plasma the fraction 2: fraction 4 activity ratio remained constant, whereas in fetal plasma the ratio decreased, a result of an increased contribution of fraction 2 to total activity. No difference in the proportion of activity associated with fractions 2 and 4 was found between the fetus directly infused with labelled phenylalanine and the fetus of an infused mother.

The 3½ hour maternal and 4 hour fetal samples had activity associated with the amino acid fraction but no activity was found in later samples. However, the diffuse nature of the amino acid fraction (4 hours elution time) resulted in low activities in the 9 separate

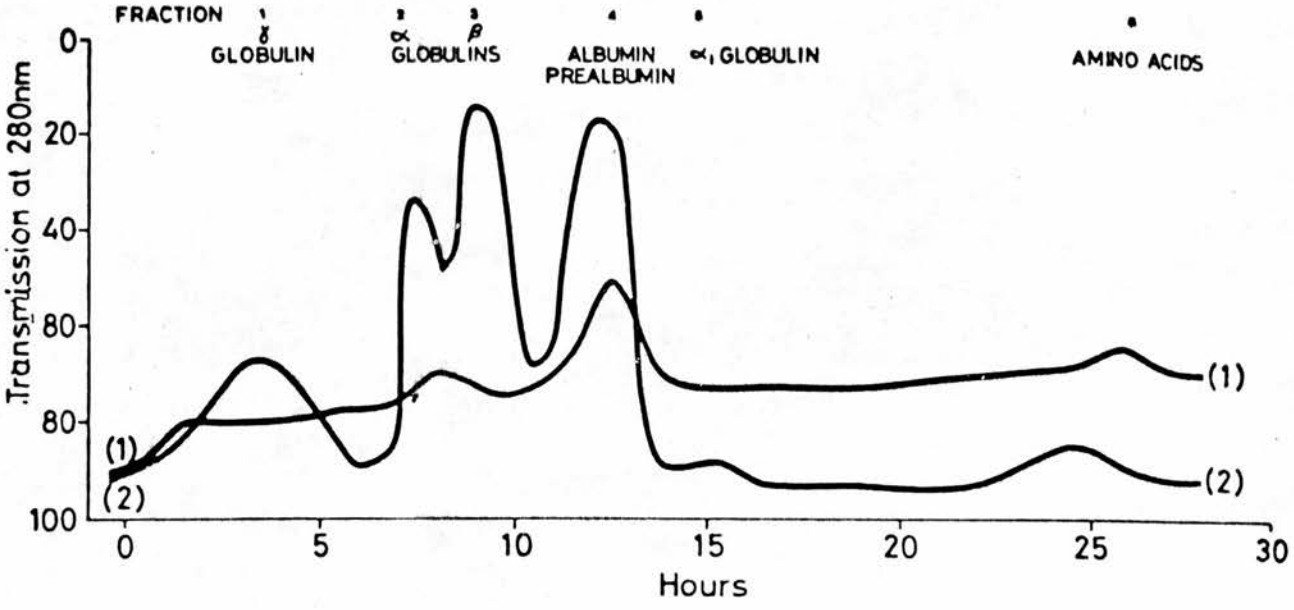


Fig. 51 - Absorption at 280 nm of eluate from Sephadex G200 column

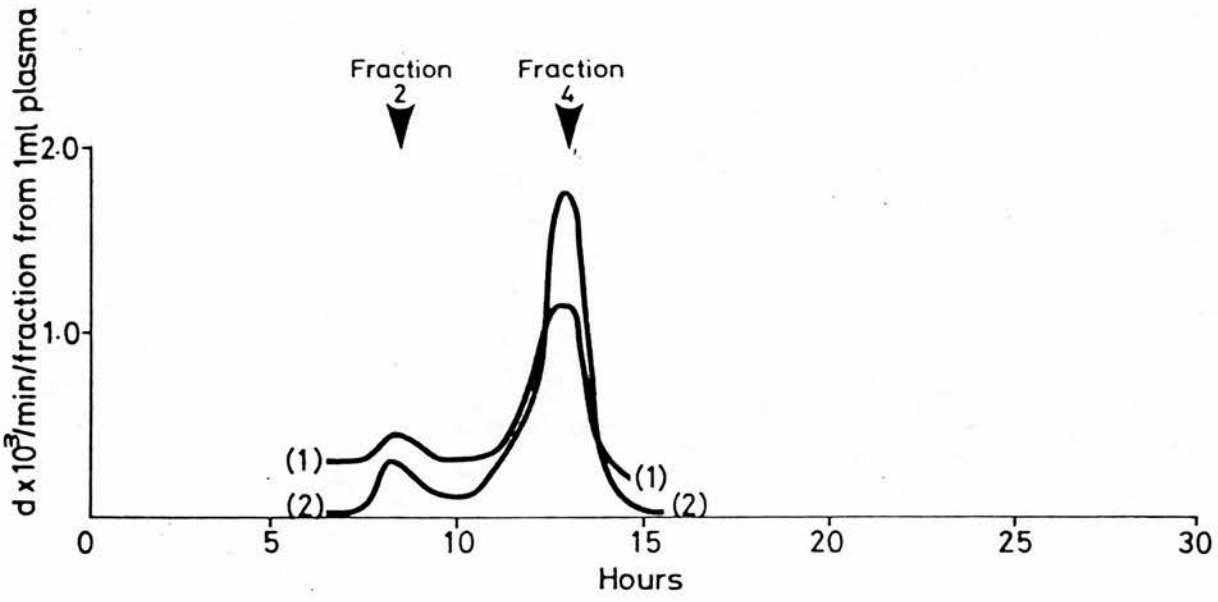


Fig. S2 - Activity in plasma protein fractions separated on G200 Sephadex

samples collected. No estimate of total free activity was therefore made.

Discussion

The albumin : and β globulin ratio was lower in maternal plasma than in the plasma of the fetus and non-pregnant adult, the concentration of albumin being reduced and that of α and β globulins increased during pregnancy[345]. No γ globulin was found in the ovine fetal plasma, γ globulin only appearing postnatally with the onset of suckling[346]. Fractions 3 and 5 were not detected in fetal plasma. These fractions might have been present below the detection limit of 280 nm monitoring of the eluate, plasmaproteins being at lower concentrations in the fetus than mother and only small volumes of fetal plasma (0.5 ml) compared with maternal plasma (3 ml) being available for Sephadex chromatography.

The relatively high activity in the albumin fraction (fraction 4) is consistent with its pool size and rate of turnover. Albumin (M.W. 69,000) is the largest single pool of plasma protein (53.6 per cent of total plasma protein in the adult sheep) and has a rapid turnover. It is synthesized exclusively in the liver. In the fetus and new born lamb the half life of mixed hepatic proteins is 0.7 days[339] compared with 6.9 days in the adult sheep[340]. However, these half lives are short when compared with those calculated from in vitro studies of human liver in which the half life was estimated at 57 days[188]. In the same study the half life of hepatic albumin was 20 days whereas that of albumin released into the medium was only

3.2 days. This is similar to that of serum albumin in the rat (3.7 days) where turnover has been calculated to be 0.027 mg/hr/g[339]. In the *in vitro* studies of Lundholm *et al*[188] a higher activity was found to be associated with a hepatic protein component which, although it reacted with albumin antisera, migrated more slowly than albumin on gel electrophoresis unless it was first subjected to mild proteolysis.

This component was therefore thought to be albumin precursor i.e. albumin with the attached proalbumin (Page 31)

In these experiments activity in the plasma albumin would be equivalent to that released into the medium. The labelling of the albumin may explain the increase in activity between hours 4 and 6 in the maternal samples of experiment C1, the higher activity still being maintained on day 8 (Table XV)

Activity in fraction 2 is probably associated with β globulins e.g. transferrin (M.W. 85,000) haptoglobulin (M.W. 85,000) and plasminogen (90,000). These have a small pool size (16.3 per cent of the total plasma protein) but a faster turnover (0.036 mg/hr/g half life of 0.5 days in rats)[339] than that of serum albumin and would therefore be preferentially labelled in constant infusion experiments of short duration.

α and β globulins have variable half lives. Together they account for 21.1 per cent of the total protein pool. Some activity in fraction 4 - the majority of which is

albumin may be that of low molecular weight α_2 globulins such as prothrombin (M.W. 62,700) but no activity was associated with either the γ or α_1 globulins such as ceruloplasmin (M.W. 150,000) the elution time of which would be similar to that of globulin. α_2 globulins of intermediate M.W. may be a component of fraction 2. No activity was found in either the fraction containing γ globulin (M.W. 170,000) which has both a small pool size (9.4 per cent of total protein in adults) and a relatively slow turnover (0.011 mg/hr/g in rats) or in fraction 5, that associated with α_1 globulins in which position fetuin, (M.W. 45,000) an α_1 globulin reported to be relatively large quantities in the plasma of ungulate fetuses would be expected to elute[346,347].

Maintenance of activity for 8 days in plasma albumin in spite of its short half life would indicate either extensive recycling of amino acids within the liver or late release of prelabelled stored hepatic albumin. Labelled albumin has been reported to be incorporated into globulins but this could have resulted from recycling. With a faster turnover of β globulins than albumin recycling or incorporation of albumin into globulins would result in a relative increase with time of activity in the globulin fraction, as was found in these experiments. The faster fetal turnover rates would enhance this effect - the ratio of fraction 2:fraction 4 being decreased by 25 per cent in the fetus at a time when the maternal ratio was unchanged.

CALCULATION

3. Flux

The flux (i.e. metabolic clearance rate) of an amino acid into and out of the total amino acid pool may be calculated from the plateau S.A. of the amino acid i.e. the point at which the quantity of labelled amino acid entering the pool is equalled by the quantity leaving.

Essential amino acids in the plasma of the fetus must have been trasported across the placenta from the maternal blood. Amino acids from proteolysis are probably rapidly utilized for protein synthesis within the cell (it has been suggested amino acids released in protein catabolism are preferentially utilized in protein synthesis[157]) and would therefore re-enter the plasma compartment. Additionally, the rapid growth and rate of protein turnover in the fetus would "bind" amino acids into protein, resulting in net amino acid flux into cells. Fetal plasma amino acids would therefore be expected to be derived directly from maternal blood via the placenta rather than from the fetal catabolism and release of amino acids into plasma. Thus the removal rate, i.e. metabolic clearance of amino acids from the placenta would represent the rate of placental transfer and fetal utilization, although not the protein synthetic rate because amino acids could also be trans-aminated, degraded or excreted via urine, skin or lung secretions.

The calculated whole body (i.e. plasma) fluxes for experiments C1, C3, C4, Ins C1, Ins C2, Ins C3, and Ins

TABLE XVI

Plasma Flux of Phenylalanine Leucine and Lysine

Experiment	Time (Hr)	Maternal SA d/min/nmol	Fetal SA d/min/nmol	Maternal* Flux µmol/hr	Fetal Flux µmol/hr	Fetal Flux µmol/kg/hr
C1	Plateau	27.5	9.8	4036	575	
C3	Plateau		193			
C4 ^{14}C	Plateau	25.5	7.3	1374		
^{3}H	Plateau		120		918	231
Ins C4		30		2220		
Ins C2	2 - 3		225		296	137
	4		173		385	178
	5		272		245	113
	9		220		302	139
Ins C3	"Plateau"		84		793	217
	max 4¼				900	247
	min 8				693	190
Ins C4	2 - 4		72		925	318
	5		170		392	135
	7		110		605	208
	7½		152		438	151
	9		64		1041	358

* Weight of sheep was approx 50 kg.

Therefore C1 C4 and Ins C4 maternal flux = 81, 27.5 and 44 µmol/kg/hr respectively

Flux calculated from the formula

$$\text{Flux} = q \frac{Si}{SA} \text{ umol/hr}$$

where q = rate of infusion (μmol/hr)

Si = SA of infused amino acid

SA = SA of plasma at plateau

^{3}H Phenylalanine SA = 1000 μCi/μmol

^{14}C Phenylalanine SA = 522 μCi/μmol

^{14}C Leucine SA = 324 μCi/μmol

^{14}C Lysine SA = 330 μCi/μmol

q ^{3}H phenylalanine (50 μCi/hr) = 49.5×10^{-3} μmol/hr

^{14}C phenylalanine (50 μCi/hr) = 95.8×10^{-3} μmol/hr

(35 μCi/hr) = 67.1×10^{-3} μmol/hr

(30 μCi/hr) = 57.5×10^{-3} μmol/hr

^{14}C leucine (30 μCi/hr) = 92.6×10^{-3} μmol/hr

^{14}C lysine (30 μCi/hr) = 90.9×10^{-3} μmol/hr

TABLE XVII

Amino Acid	Data of Lemons et al[124]				Infusion data
	uv ua amino acid concentration µmol/l		Calculated N_2^+ transfer µg/kg/day		Calculated N_2^+ transfer µg/kg/day
	Range	x	Range	x	
Lysine	-40-+50	+9	-0.271-+0.339	+0.060	+0.213
Leucine	-10-+60	+22	-0.034-+0.203	+0075	+0.073
Phenylalanine	-10-+0.40	+12	-0.034-+0.136	+0041	+0.060

* N_2 transfer µg/kg/day = uv - ua (µg/N_2/ml) X blood flow (168 ml/kg/min) X 1440

** N_2 transfer = µgN_2 flux/kg/hr X 24

+ N_2 = protein nitrogen

x = mean

C4 are given in Table Flux is normally calculated from plateau SA, but in experiments which included a period of insulin infusion the flux has also been calculated from the SA of the samples taken during and after stopping the insulin infusion, i.e. at a time when the metabolic equilibrium of the fetus was disturbed. Although total maternal flux was obviously greater than that of the fetus, expressed on a body weight basis, the fetal flux is greater. The faster flux is consistent with the greater metabolic activity of fetal tissues, both anabolic and catabolic proceeding at a faster rate in the fetus than the mother.

The leucine flux in experiment Ins C3 of 217.3 µmol/kg/hr was less than that reported for newborn Clun lambs (689 - 950 µmol/kg/hr)[339]. Anaesthesia, differences in maturity and environment (extra uterine as opposed to intrauterine), species and individual variations could account for the discrepancy. This is supported by the fact that there is close agreement between transfer rates based on the flux measurements from these experiments and the net placental transfer of amino acids to the fetus calculated from the data of Lemons et al[124]. In Lemons' experiments fetal blood flow and amino acid concentrations in umbilical venous and arterial blood were measured in unstressed fetal lambs of 118 - 146 days gestation, enabling transfer rates to be calculated using the uv:ua difference (in the calculation of table XVII the range of uv:ua difference was derived from published figures). A comparison of the calculated transfer rates is shown in Table XVII .

Calculated values for lysine transfer were 0.060 and 0.213 µg/kg/day for leucine transfer 0.075 and 0.073 µg/kg/day and for phenylalanine 0.041 and 0.060 µg/kg/day based on the data of Lemons et al and these experiments respectively. Placental transfer of some amino acids, including leucine and phenylalanine is considerably in excess of the estimated growth requirements based on carcass analysis. This would suggest transamination and oxidative degradation of essential amino acids does occur in the fetus, this possibly varying with gestation as changes in uv:ua concentration differences have been found towards term[121]. With the exception of aspartic acid (which is closely associated with amidation to asparagine) lysine is the only amino acid in which the estimated requirements from carcass analysis are in close agreement with the calculated transfer rate. As lysine is not transaminated, this could imply that most of the difference between the two types of estimation is due to degradation rather than transamination.

The reduced flux and decreased amino acid concentration associated with insulin infusion might reflect increased net protein synthesis. The decrease in flux could suggest that placental transfer had decreased, and that any net increase in protein synthesis might be the result of reduced catabolism rather than increased synthesis. It also supports the concept that insulin stimulation of amino acid uptake from the fetal plasma into the placenta is more powerful than the passive diffusion of amino acids from the placenta to the fetal blood

TABLE XVIII

Infant	Delivery	Birthweight (g)	Gestation (wk)	Sex	Post Natal* Age (days)	Day IV started	Schedule	Clinical Complications
Fo	SVD	1240	34	F	13	2	1	Severe IRDS
Gt	SVD	1162	27	M	2	2	1	IRDS
Tw	KFD	1332	33	M	1	1	1	IRDS
An	SVD	1588	29	F	2	2	2	Mild IRDS
Ad	SVD	2637	35	M	4	4	2	IRDS, 2nd twin
Bl	SVD	1120	28	M	26	6	2	IRDS
Ga	SVD	2600	34	M	3	2	2	IRDS, cardio-respiratory arrest
Gr	CS	1729	31	F	5	2	2	Maternal diabetes

245

+ at entry into study:

SVD = spontaneous vertex delivery

KFD = Keillands forceps delivery

CS = Caesarean section

IRDS = ideopathic respiratory distress syndrome

CHAPTER V

Neonatal Studies

Both maternal and fetal insulin secretion appear to influence fetal growth (see chapter I section 5 p 64). In the newborn infant adaptation of insulin release to altered and fluctuating plasma concentrations of glucose and amino acids as a result of both a changed and intermittent dietary intake is necessary for continuing post natal weight gain. The initial response of insulin secretion to a change in plasma glucose and amino acid concentration is delayed, but becomes brisker after a few days. As insulin facilitates movement of amino acids into cells and increases net protein synthesis, administration of exogenous insulin may enchance utilization of parenterally infused amino acids and thereby encourage protein synthesis in the preterm infant.[38],

Plasma amino acid concentrations were measured in parenterally fed preterm infants before, during and after the infusion of insulin. No assessment of the role of intravenous feeding or the suitability of the aminoacid solution used was made, only the changes in concentration on and off insulin being studied.

Materials and Methods

Eight infants were entered into the study and these were randomly allocated to one of two insulin infusion schedules (see following page). Clinical details of the infants and the schedule to which the infant was allocated are given (on the opposite page).

All infants were parenterally fed Vamin 7%*, 10% dextrose/electolyte/vitamin solution* and Intralipid 20% were infused at 60, 40 and 10 ml/kg/day respectively for Tw, Gt, Ad, An and Ga and at 30, 90 and 10 ml/kg/day for infants Bl, Fo and Gr, providing Kcal/kg/day.

Insulin to give 0.2 iu/kg/hour was added to the dextrose solution together with low salt albumin.

* see appendix[13] for composition

albumin (200 mg/100ml dextrose/electrolyte/vitamin solution), according to one of the two schedules given below:-

1) C/I/C

Control 24 hour period (Vamin, cocktail plus albumin, Intralipid only)

24 hour insulin infusion (plus Vamin, cocktail plus albumin, Intralipid)

Control 24 hour period

2) I/C/I

24 hour insulin infusion

Control 24 hour period

24 hour insulin infusion

Blood samples were taken (via an indwelling catheter) at 0,3,24, 27, 48, and 72 hours from all infants who completed the trial period or at these time intervals until withdrawal from the study because of their clinical condition. Additional samples were taken at 12, 36 and 60 hours from infants Ga Gt and Tw and a further 24 hour period was completed in infants An and Tw.

In addition to samples for biochemical analysis, blood glucose was monitored using dextrostix strips and heel prick blood samples every 3 hours during insulin infusion.

Blood glucose, plasma amino acids, total lipid and non-esterfield fatty acid (NEFA) concentrations, osmolality, pH and blood gas status were measured.

24 hour urine samples were collected (unless the perineal skin condition precluded this) to correspond with the 24 hour infusion periods. Volume, osmolality and amino acid concentrations were measured and a labstix test on fresh urine performed.

Table N1

Blood gas, acid-base status and plasma osmolality of infants during the study.

Infant	Day	* Time	pH	H+ nmol/l	pCO_2 KPa	pO_2 KPa	HCO_3 mmol/l	BE mmol/l	Osm mosm/l
Fo	1	11.10	7.31	49	8.1	8.2	30.0	+2	291
	2	11.00	7.32	48	8.1	7.3	31.0	+2	292
	2	19.25	7.16	71	8.5	9.4	21.5	-9	
	3	11.00	7.43	37	9.7	7.4	49.0	+19	293
	3	14.35	7.40	40	6.7	14.0	31.0	+5	
	4	11.00	7.38	38	10.0	6.7	44.5	+15	
Gt	1	10.00	7.28	52	6.5	5.3	22.5	-4	281
	2	10.00	7.31	49	6.5	7.3	24.0	-2	287
	2	21.30	7.26	55	6.6	10.7	21.5	-5	287
Tw	1	11.00	7.30	50	5.5	8.8	20.0	-5	281
	2	11.00	7.34	46	5.3	6.6	21.0	-4	308
	3	11.00	7.30	50	5.5	8.8	20.0	-6	295
	4	11.00	7.32	48	3.7	7.3	19.0	-6	300
An	1	11.00	7.36	44	5.4	10.8	22.5	-2	
	2	11.00	7.32	48	5.4	10.0	20.5	-4	298
	3	11.00	7.26	55	3.2	9.3	17.0	-9	330
	4	11.00	7.35	45	4.6	11.6	19.0	-5	305
	5	11.00	7.34	46	5.0	9.4	20.0	-4	307
Ad	1	11.00	7.35	45	6.8	16.7	28.5	+2	295
	2	08.30	7.30	49	6.8	12.6	26.0	-1	
	2	11.00	7.14	72	9.0	14.2	22.5	-8	297
	2	19.00	7.35	45	5.9	12.3	24.5	-1	
	3	11.00	7.38	42	6.1	13.0	27.0	+2	334
	4	11.00	7.42	38	5.4	19.5	27.0	+2	326

Table N1 (cont'd)

Infant	Day	Time*	pH	H+ nmol/1	pCO_2 KPa	pO_2 KPa	HCO_3 mmol/1	BE mmol/1	Osm mosm/1
B1	1	11.00	7.32	48	7.7	7.2	29.5	+2	293
	2	11.00	7.34	46	7.4	8.2	29.5	+2	302
	3	11.00	7.26	54	7.8	9.4	26.5	-2	287
	4	11.00	7.38	42	8.5	6.3	38.0	+10	279
Ga	1	10.00	7.36	44	5.0	9.1	21.0	-3	
	2	10.00	7.24	58	7.3	3.6	22.5	-5	
Gr	1	11.00	7.29	51	6.4	8.1	22.0	-4	
	2	11.00	7.35	45	6.6	8.4	23.0	-2	

* At time of changeover in schedule. Additional values given if either a change in one of the values occured or the routine sample was not representative of the 24 hour period.

TABLE N2

Urine volume, osmolality and "labstix" reading.

Infant	Day of Trial	Volume ml/day	Osmolality mosm/kg	Labstix result
Fo	1	70+	163	-ve
	2	218	191	-ve
	3	94	189	-ve
Gt	1	40	461	-ve
	2	15	483	-ve
Tw	1	100	137	-ve
	2	49	352	-ve
	3*	(9+34+74) (117)	(436+366+218) (278)	-ve
	4	115	224	-ve
An	1	45	288	-ve
	2	19.5**	346	-ve
	3	discontinued		-ve
Ad	1	243	326	-ve
	2	145	329	-ve
	3	143	326	-ve

TABLE N2 (cont'd)

B1	1	270	233	-ve
	2	238	245	-ve
	3	183	223	-ve
Ga	1	103	437	-ve
Gr	1	85	280	-ve
	2	97	268	-ve

* (separate collections) (total)

** large losses

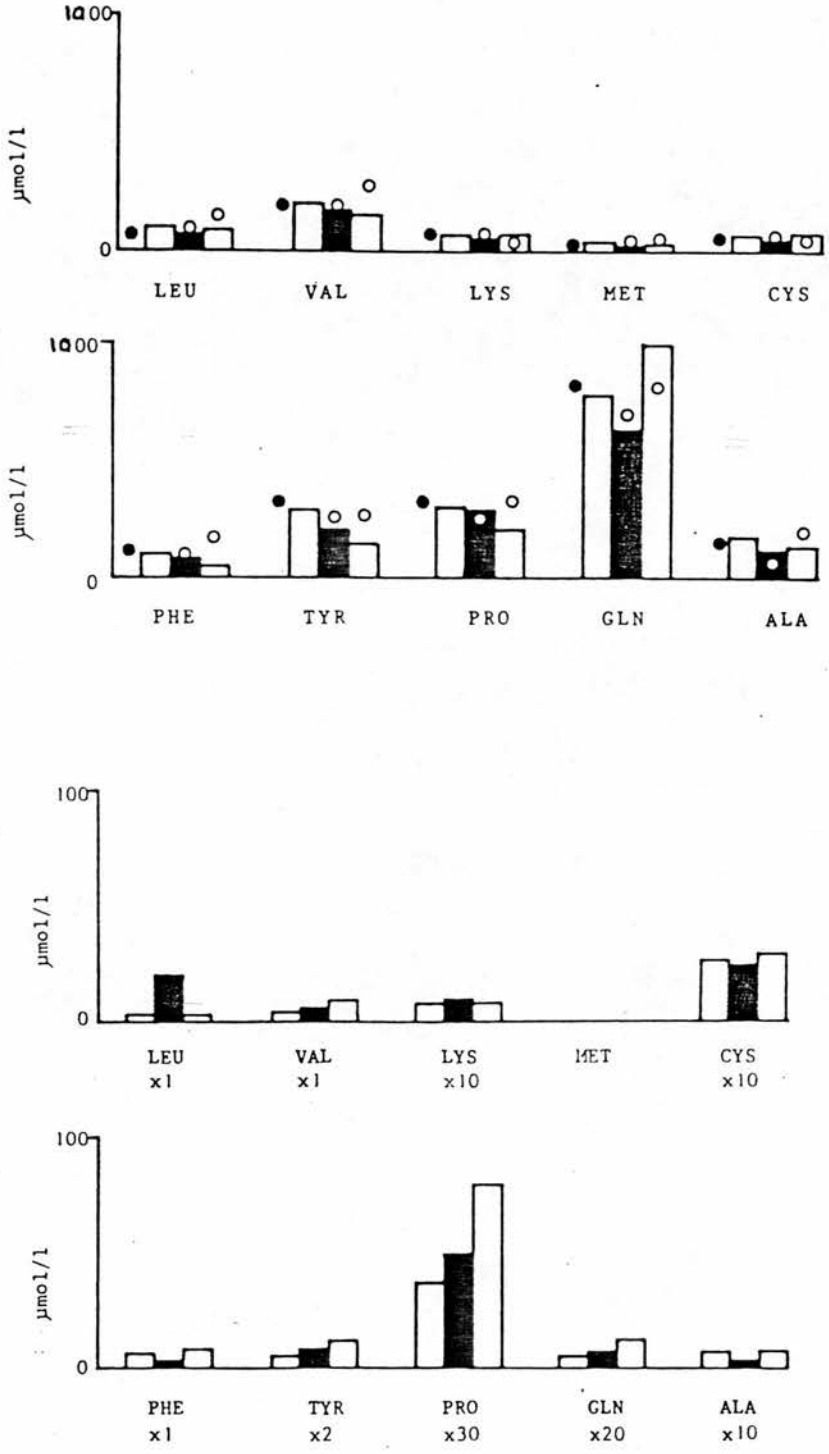
RESULTS

Blood, pH and gas partial pressures and plasma osmolality results are given in Table N1.

Urine volume, osmolality and labstix results are summarised in Table N2.

Blood glucose, total lipid, NEFA and selected amino acid results are presented in histogram form. The amino acids illustrated in the histogram represent different functional and transport groups (See p 264)

Measurements made at zero hours (●) and at 3 hours (represented by open circles or solid white circles within the black histograms) and 24 hours (histograms) after a change from or onto insulin infusion are displayed in the following figures. Periods of insulin infusion are represented by solid black histograms and periods off insulin by open histograms.

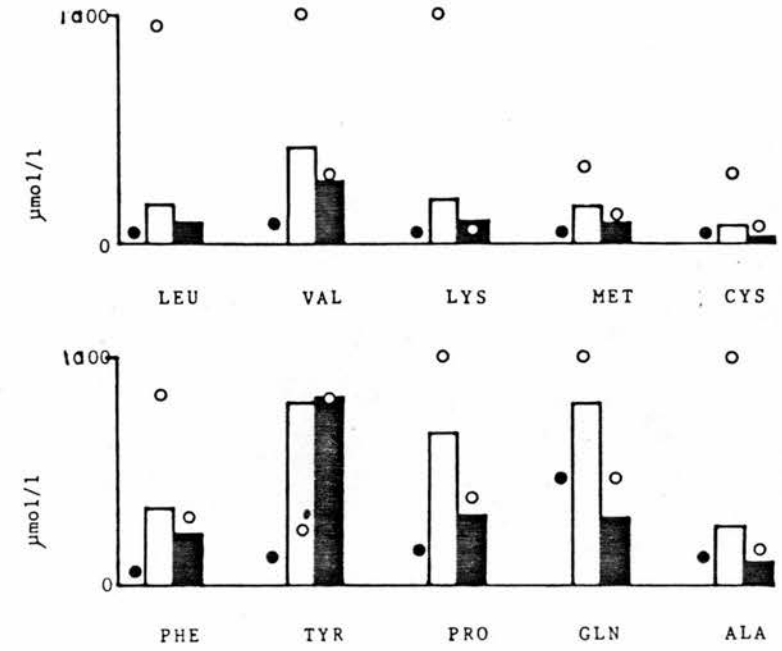


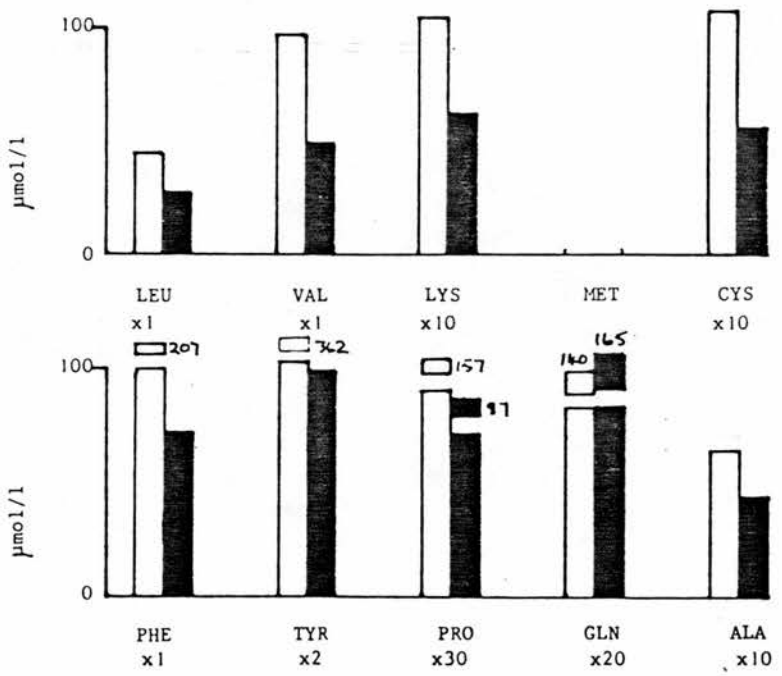
Infant Fo: Plasma (above) and urine (below) amino acid concentrations during ■ and off □ insulin infusion.

(0 - 3 hours after changeover)

(□ - 24 " " ")

X - Multiplication Factor



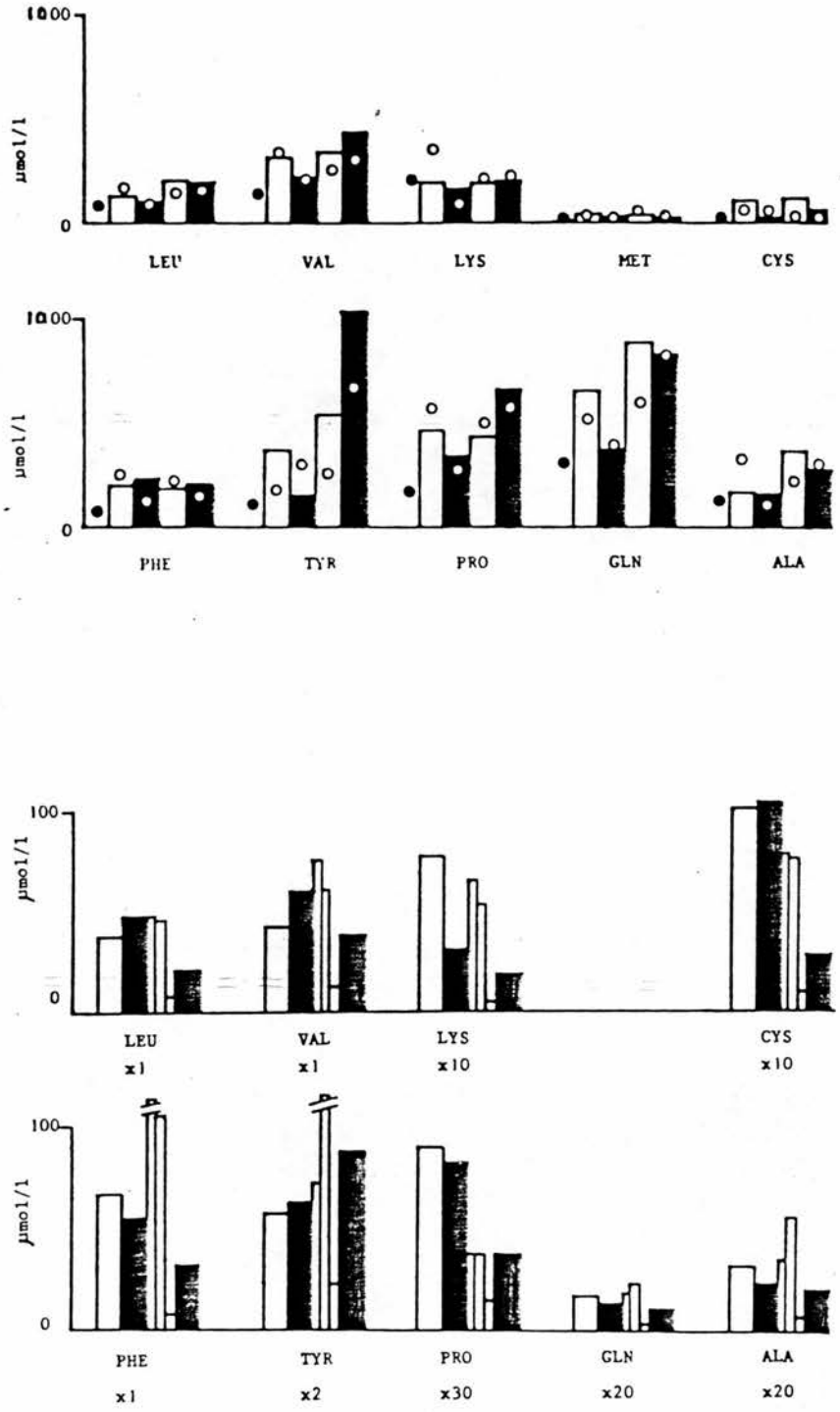


Infant Gt: Plasma (above) and urine (below) amino acid concentrations during ■ and off □ insulin infusion.

(0 - 3 hours after changeover)

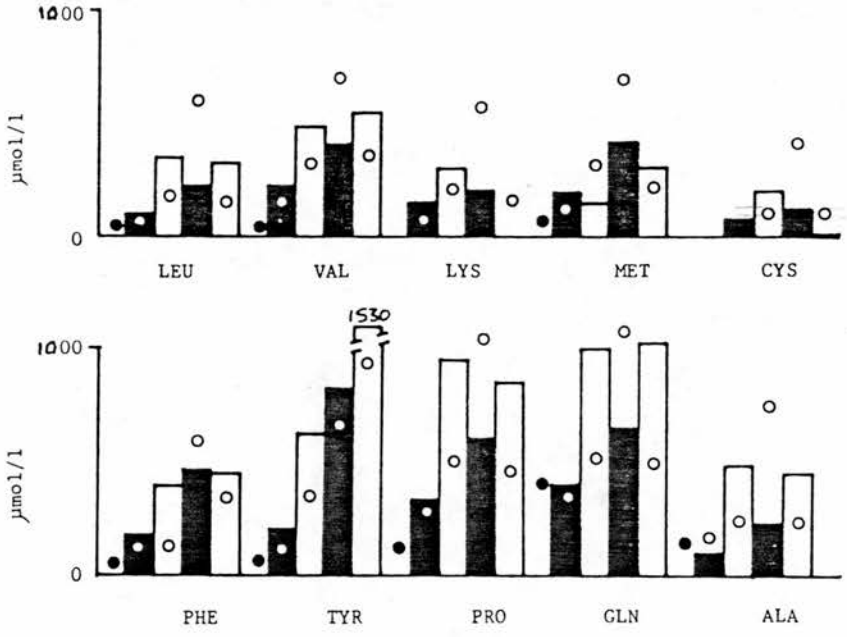
(□ - 24 " " ")

X - Multiplication Factor



Infant Tw: Plasma (above) and urine (below) amino acid concentrations during ■ and off □ insulin infusion.

(0 – 3 hours after changeover)
(□ – 24 " " ")
X – Multiplication Factor

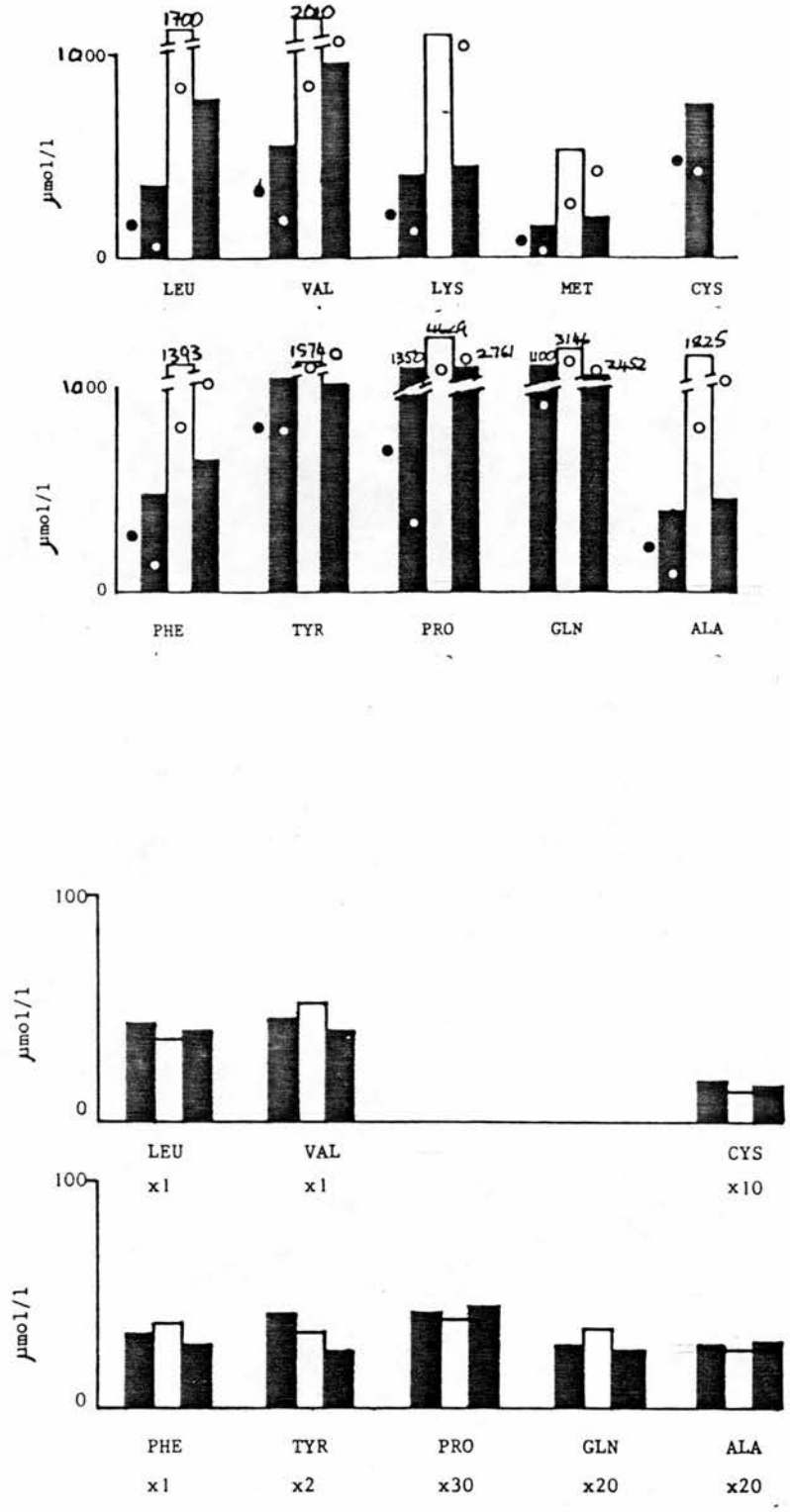


Infant An: Plasma amino acid concentrations during ■ and off □ insulin infusion.

(0 - 3 hours after changeover)

(□ - 24 " "

X - Multiplication Factor

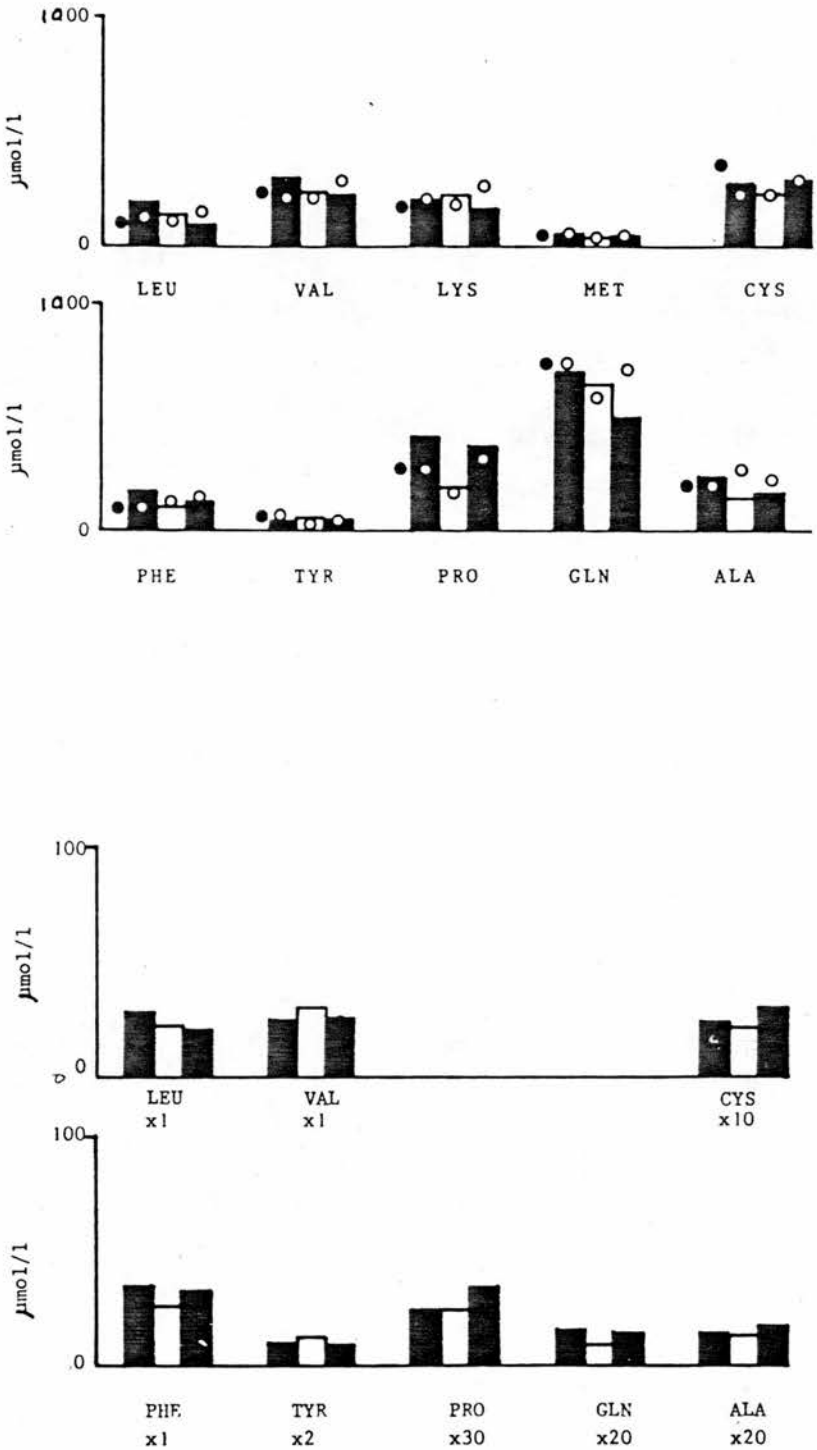


Infant Ad: Plasma (above) and urine (below) amino acid concentrations during ■ and off □ insulin infusion.

(0 - 3 hours after changeover)

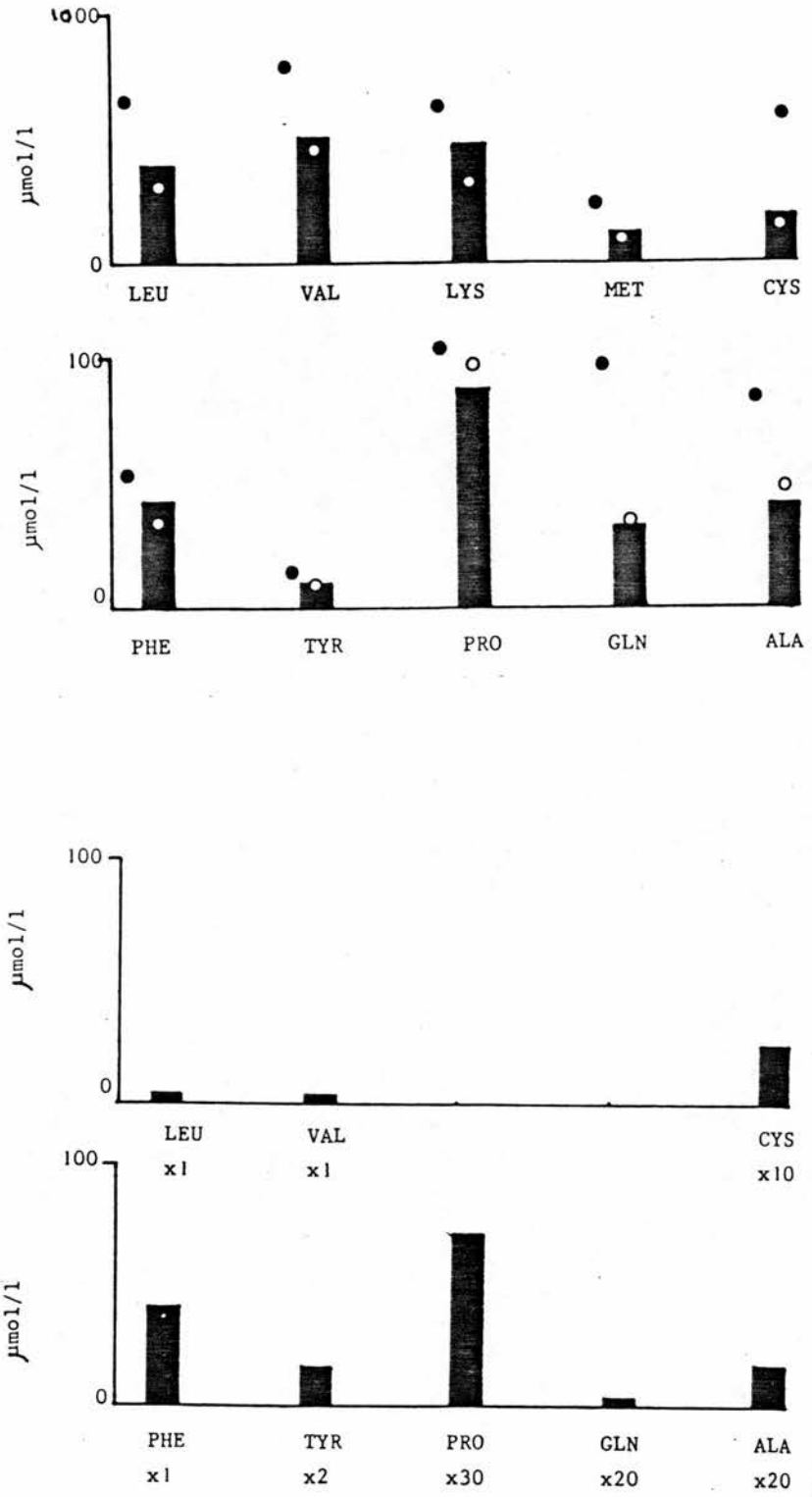
(□ - 24 " " ")

x - Multiplication Factor



Infant B1: Plasma (above) and urine (below) amino acid concentrations during ■ and off □ insulin infusion.

(0 - 3 hours after changeover)
(□ - 24 " " ")
X - Multiplication Factor

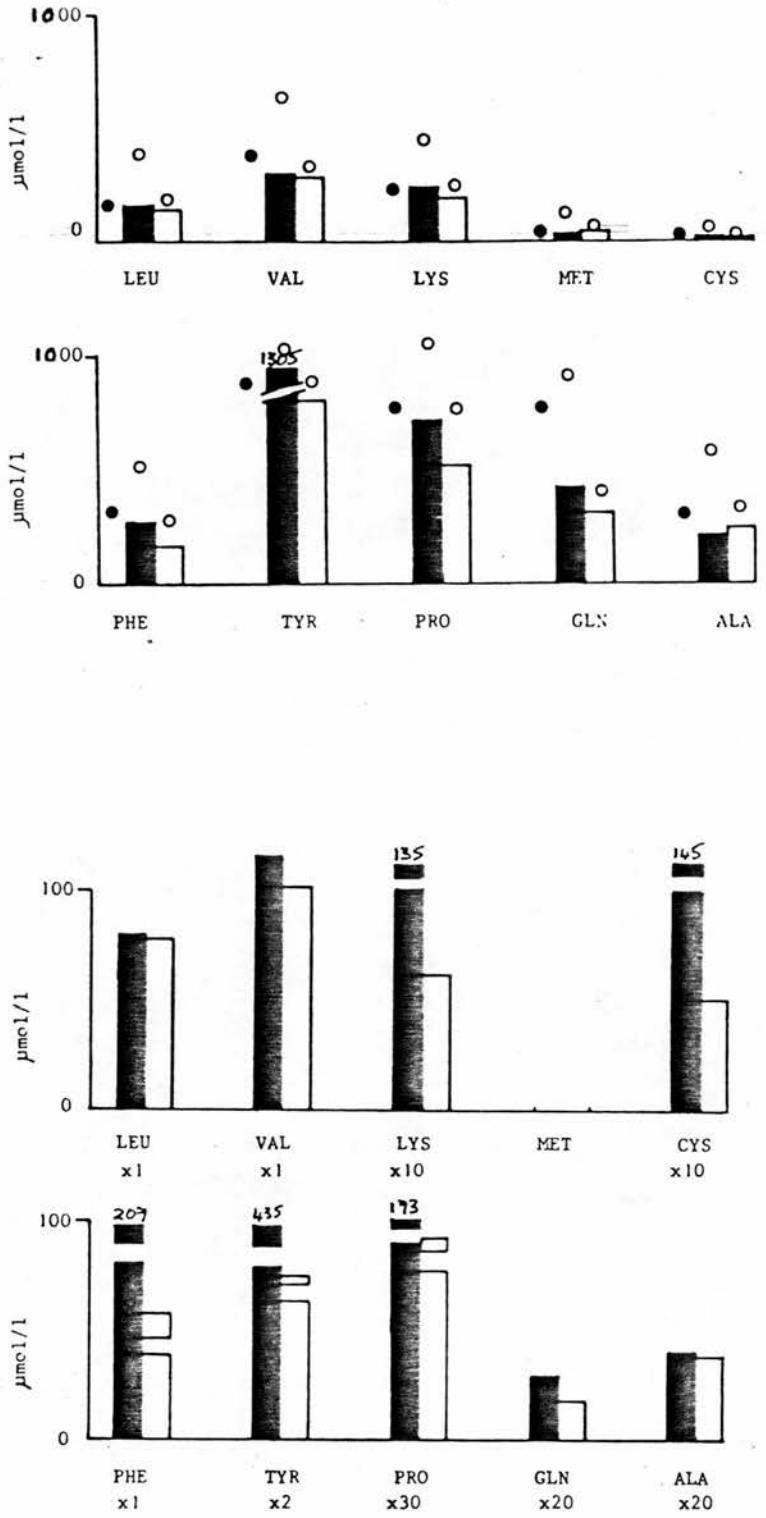


Infant Ga: Plasma (above) and urine (below) amino acid concentrations during ■ and off □ insulin infusion.

(0 - 3 hours after changeover)

(□ - 24 " " ")

X - Multiplication Factor

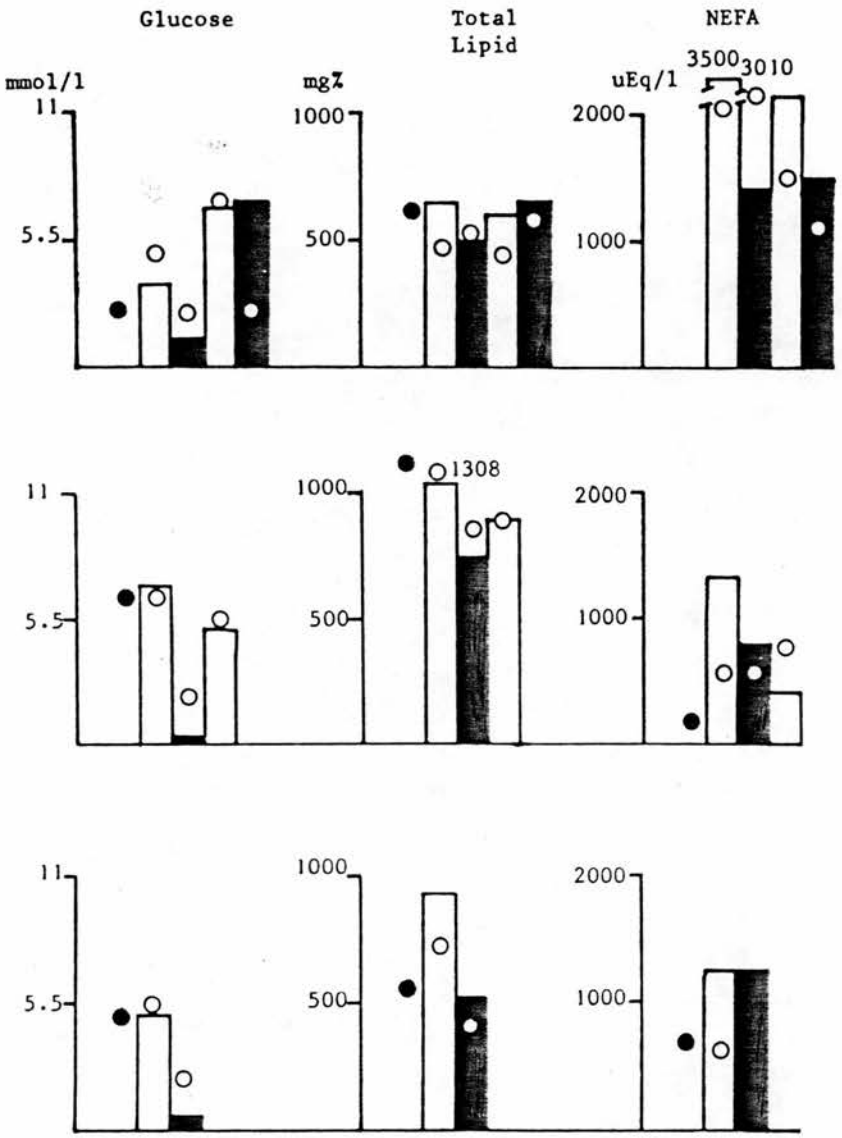


Infant Gr: Plasma (above) and urine (below) amino acid concentrations during ■ and off □ insulin infusions.

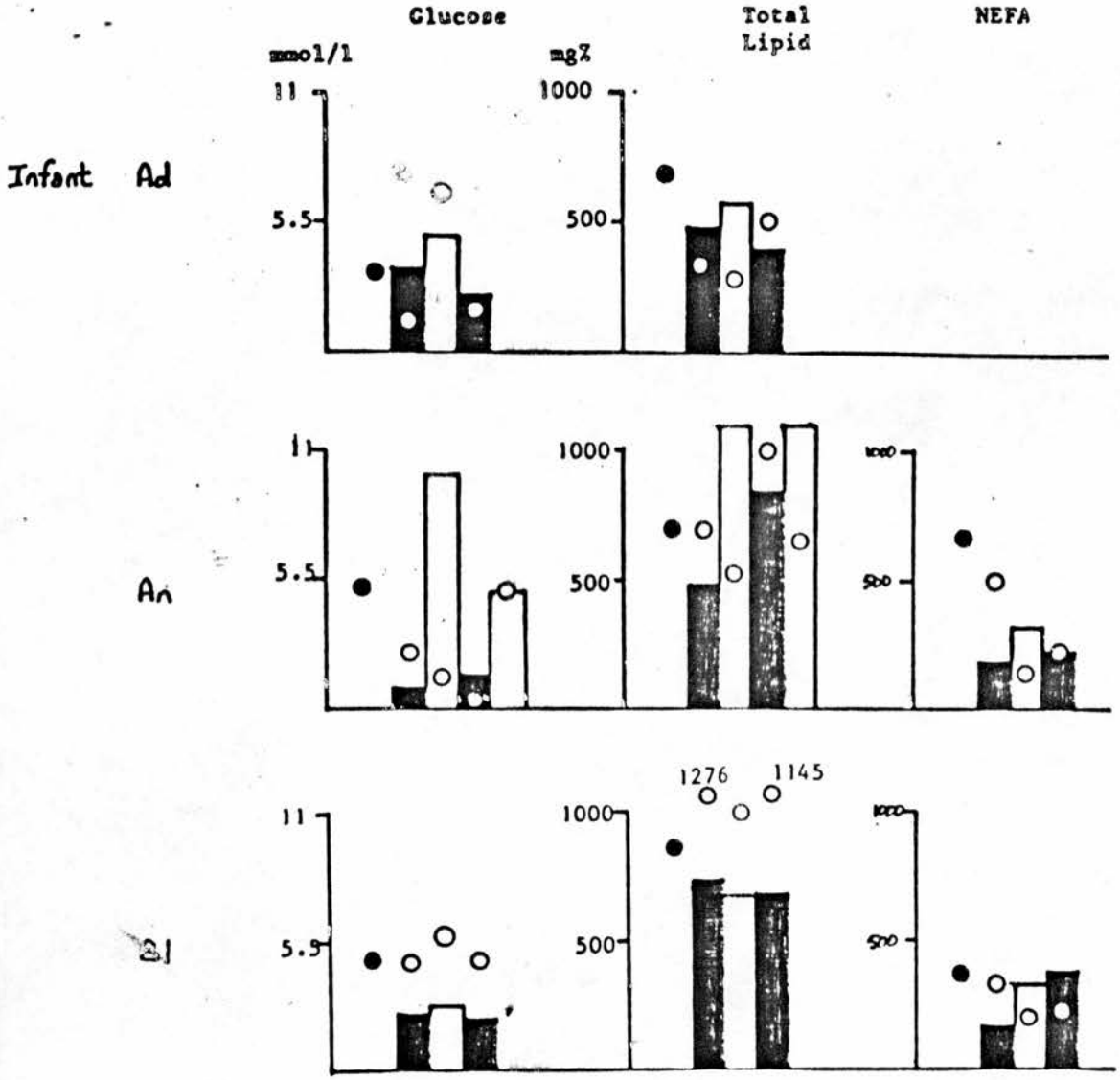
(0 - 3 hours after changeover)

(□ - 24 " " ")

X - Multiplication Factor



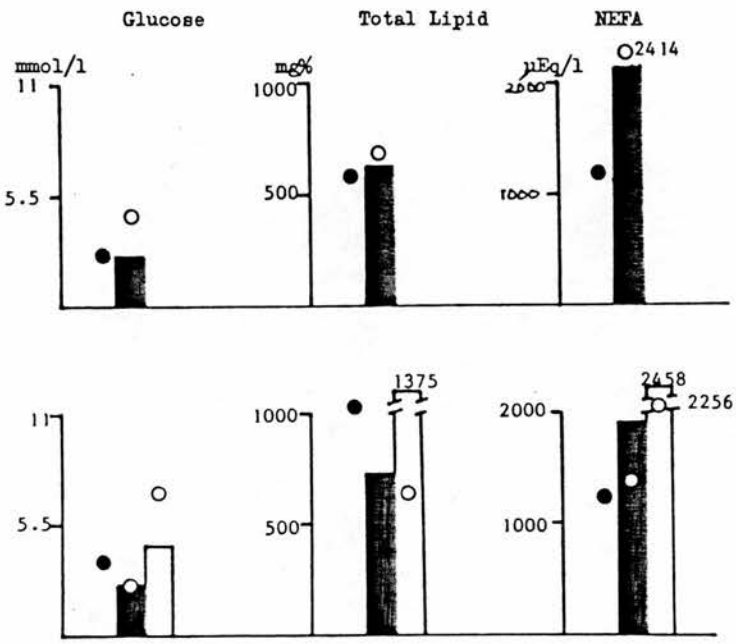
Blood glucose, total lipid and NEFA concentrations during (■) without (□) insulin infusion.



Blood glucose, total lipid and NEFA concentrations during (■) without (□) insulin infusion.

Infant Ga

Gr



Blood glucose, total lipid and NEFA concentrations during (■) without (□) insulin infusion.

Gluconeogenic amino acids - alanine and glutamine

Branched chain ketogenic acids - valine* and leucine*

Liver metabolised amino acids - phenylalanine*, tyrosine*

Cystine* methionine*.

Muscle "metabolised" amino acids - proline - leucine*, valine*.

"Inert" - limited metabolism - lysine*.

An indication of the effect on these amino acid concentrations when insulin infusion is either started or stopped in shown in Tables N3 and N4.

Discussion

Fetal plasma amino acid concentrations are greater than those of neonatal plasma (See Chapter 1 , Section1) and therefore it could be argued that for the preterm infant high plasma amino acid concentrations are more "normal" than those of full-term infants[39]. During the early period of this study Vamin was infused at a high rate but subsequent clinical practice has reduced the rate to give a ratio of 9:3:2 (Cocktail:Vamin:Intralipid) - that used for the last three infants included in the study. Theoretically the infusion of exogenous insulin would be expected to increase cellular uptake of amino acids and thereby reduce plasma free amino acid concentrations (see page 45). However, the simultaneous infusion of large quantities of crystalline L amino acids may cause an increase in plasma free amino acid concentrations and this in turn may stimulate endogenous insulin release - thus obscuring responses to the infused insulin. Also if insulin precipitously decreased plasma glucose concentrations,

* essential amino acids in newborn preterm human infant.

TABLE N3

Effect of infusing insulin on plasma free amino acid concentrations

	3 Hours post Insulin			24 Hours post insulin		
Amino Acid	No. of analyses	No. decreased*	% decreased	No. of analyses	No. decreased*	% decreased
Glycine	11	9	82	11	7	64
Serine	11	4	36	11	6	45
Glutamine	11	7	64	11	9	73
Taurine	10	5	50	9	3	33
Leucine	11	6	55	11	7	64
Isoleucine	11	6	55	11	5	45
Valine	11	7	64	11	7	54
Methionine	11	6	55	11	4	36
Phenylalanine	11	6	55	11	4	36
Tyrosine	11	4	36	11	5	45
Alanine	11	7	64	11	7	64
Lysine	11	6	54	11	6	54
Proline	11	5	45	11	6	54
Total	142	78	55	141	76	54

* change greater than 10%

TABLE N4

Effect of terminating insulin infusion on plasma free amino acid concentrations

	3 Hours after termination			24 Hours after termination		
Amino Acid	No. of analyses	No. increased	% increased	No. of analyses	No. increased	% increased
Glycine	6	3	50	6	5	83
Serine	6	4	66	6	3	50
Glutamine	6	4	66	6	5	83
Taurine	5	3	60	5	3	40
Leucine	6	4	66	6	4	66
Isoleucine	6	3	50	6	3	50
Valine	6	4	66	6	4	66
Methionine	6	4	66	6	4	50
Phenylalanine	6	3	50	6	2	33
Tyrosine	6	5	83	6	5	83
Alanine	6	5	83	6	4	83
Lysine	6	3	50	6	3	50
Proline	6	4	66	6	4	66
Total	77	49	65	77	49	64

* change greater than 10%

glucagon secretion might be stimulated and amino acids - particularly alanine and glutamine - may be mobilised and utilised as energy sources resulting in a changed amino acid profile.

In these studies a significant decrease ($p < 0.001$) in blood glucose concentration, as judged by 24 hour values, did occur during insulin infusion, and all babies recorded dextrostix readings of 25 mg/100 ml or less on at least one occasion when on insulin. Three hours after changing regimes there was little correlation between blood glucose and total lipid but at 24 hours the decrease in glucose on infusing insulin was always accompanied by a decrease in the total lipid concentration. Similarly the increase in blood glucose at 24 hours after withdrawing insulin resulted in increased total lipid concentrations. The correlation coefficient $r = 0.566$ of blood glucose and total lipid was not significant for the group as a whole nor was there correlation with either the group on or the group off insulin infusion. However, although the relative concentrations of plasma glucose and lipid vary with individual infants, the close relationship between falling blood glucose and falling total lipid concentrations suggest that the infants begin to utilise or accelerate the metabolism of fat to meet their metabolic energy requirements. Transition from carbohydrate to fat metabolism is faster in children than in adults[348]. In term infants with relatively large adipose stores but small carbohydrate reserves this is very important. In the preterm infant with smaller fat stores, infusion of intralipid may permit

the same adjustment to take place when glucose concentrations fall as a result of raised insulin concentrations. No correlation between non-esterified fatty acids and either blood glucose or total lipid was found - possibly because the proportion of Intralipid being utilised for energy or deposition was changed rapidly. If energy requirements are compromised, it is unlikely that optimum protein synthesis is possible and although amino acids may be encouraged to enter the cell on infusing insulin it may well be they then enter the catabolic pathways - and the second protein synthetic stimulatory action of insulin or its inhibitory effect on catabolism is abolished. Improved control of Intralipid and dextrose could therefore increase protein synthesis on the same amino acid regime, Chen et al[349] having previously demonstrated caloric supply influences the plasma amino acid profile. During these studies alanine and glutamine, the two amino acids most sensitive to caloric deficits behave in a manner similar to other amino acids, so that although catabolic pathways may be operating in the cells, it suggests that mobilisation of amino acids is limited. From the histograms it can be seen that there was a general decrease in plasma glucose and amino acids after infusion of insulin, although this was not true for all amino acids in all infants. Few significant changes in plasma free amino acid concentrations were found during periods on and off insulin. Those found to change significantly were:-

Glycine ($P < 0.5$) - Tw

Glutamine ($P < 0.5$) - Tw and Fo

Valine ($P < 0.01$) - Fo

TABLE N5

Effect of infusing insulin on plasma amino acid concentrations of the two infusion schedules.

Amino Acid	No. of Plasma Amino Acids Decreased *							
	3 Hours Post				24 Hours Post			
	1st Insulin Infusion of Schedule		2nd Insulin Infusion of Schedule		1st Insulin Infusion of Schedule		2nd Insulin Infusion of Schedule	
	1	2	1	2	1	2	1	2
Glycine	3/3	4/4	1/1	1/3	2/3	1/4	1/1	3/3
Serine	2/3	2/4	0/1	0/3	2/3	1/4	0/1	3/3
Glutamine	3/3	3/4	0/1	1/3	3/3	3/4	0/1	3/3
Taurine	2/3	2/4	0/1	0/2	1/3	1/3	0/1	1/2
Leucine	3/3	2/4	0/1	1/3	3/3	1/4	0/1	3/3
Isoleucine	3/3	2/4	0/1	1/3	1/3	1/4	0/1	3/3
Valine	3/3	3/4	0/1	1/3	3/3	1/4	0/1	3/3
Methionine	3/3	2/4	0/1	1/3	2/3	1/4	0/1	1/3
Phenylalanine	3/3	2/4	0/1	1/3	2/3	1/4	0/1	1/3
Tyrosine	2/3	1/4	0/1	1/3	2/3	2/4	0/1	1/3
Alanine	3/3	2/4	1/1	1/3	2/3	2/4	1/1	2/3
Lysine	2/3	2/4	0/1	2/3	2/3	1/4	0/1	3/3
Proline	2/3	1/4	1/1	1/3	3/3	1/4	1/1	2/3
Total	34/39	28/52	3/13	12/38	28/39	17/51	2/13	29/38

TABLE N6

Effect of Terminating Insulin Infusion on Plasma Amino Acid Concentrations.

Amino Acid	No. of Plasma Amino Acids Increased*			
	3 Hours post termination		24 Hours post termination	
	1st Infusion Period in Schedule	2nd Infusion Period in Schedule	1st Infusion Period in Schedule	2nd Infusion Period in Schedule
	1 2	1 2	1 2	1 2
Glycine	1/2 2/3	0/1	2/2 2/3	1/1
Serine	2/2 2/3	0/1	0/2 2/3	1/1
Glutamine	2/2 2/3	0/1	2/2 2/3	1/1
Taurine	1/2 1/1	1/1	1/2 1/1	1/1
Leucine	2/2 2/3	0/1	1/2 2/3	1/1
Isoleucine	1/2 2/3	0/1	0/2 2/3	1/1
Valine	2/2 2/3	0/1	1/2 2/3	1/1
Methionine	2/2 2/3	0/1	2/2 1/3	1/1
Phenylalanine	1/2 2/3	0/1	0/2 2/3	0/1
Tyrosine	2/2 2/3	1/1	1/2 3/3	1/1
Alanine	2/2 3/3	0/1	1/2 2/3	1/1
Lysine	1/2 2/3	0/1	0/2 2/3	1/1
Proline	2/2 2/3	0/1	1/2 2/3	1/1
Total	21/26 26/37	2/13	11/26 25/37	12/13
P	0.05 0.05		0.05	0.05

* Change greater than 10%

Phenylalanine ($P < 0.5$) - Tw

Tyrosine ($P < 0.01$) - Tw

($P < 0.05$) - Ga

The large variability in plasma amino acid concentrations in any one infant when infused with amino acids during the 24 hour periods on or off insulin may obscure insulin induced changes. Adaptation to the higher levels of insulin and the half-life of insulin after its withdrawal could well contribute to the large standard deviations of plasma amino acid concentrations found even in individual infants. It is possible that in utero conditions of Gr, an infant of a diabetic mother, might have affected the postnatal response to insulin (page 35), therefore baby Gr has been excluded from the combined data in Tables N3 - 6.

From Table N3, in which the direction of change in concentration between the last sample taken before starting insulin and the samples taken 3 or 24 hours later is examined, it can be seen that there is no trend for insulin to decrease plasma amino acid concentrations although it should be noted that in more than half of the samples plasma amino acids were decreased and in very few cases was there an actual increase. However, if the two regimes - C/I/C and I/C/I are examined separately (Table N5) it can be seen that when insulin is infused after an initial 24 hour period of Vamin alone the decrease is significant at the 0.1 per cent level (X^2 test). Immediate infusion of Vamin with insulin obscures any response of amino acids to insulin in the first 3 hours of the second schedule I/C/I.

At 24 hours after the start of the first insulin infusion, the number of occasions on which amino acids are decreased is no longer significant in either group - both groups having fewer decreased concentrations at this time than at 3 hours. If a second period of insulin infusion was given, in the I/C/I regimen although a smaller proportion were decreased during the first 3 hours, an increased proportion - significant at the 5 per cent level - were decreased by 24 hours. Only one infant on the C/I/C regimen had a second period of insulin - during which time amino acid concentrations were little changed from the previous 24 hour values.

It therefore appears that if amino acids and insulin are initially infused together, the increased input of amino acids is of greater importance in determining plasma amino acid concentrations than the presence of insulin, which would decrease the plasma amino acid concentrations. However, insulin did reduce the initial increase in plasma amino acid concentrations in the I/C/I regimen and decreased amino acid concentrations after the infusion of insulin in the C/I/C regimen and after the second insulin infusion of the I/C/I regimen. The lower infusion rates of amino acids for Bl, Fo and Gr did not alter the overall response of amino acid concentrations to insulin infusion although the changes were smaller. Previous studies have reported hyperaminoacidaemia on withdrawing insulin from infants on prolonged intravenous therapy. In this study, in both groups of infants, a significant number (P 0.05) of increased amino acid concentrations were noted (Table N6) in the blood samples taken 3 hours after discontinuing

insulin compared with the last sample taken during insulin therapy. The same was true for samples taken at 24 hours in infants on the I/C/I regime. When data for all periods in both groups of infants was combined - Table N4), the slow increase in plasma amino acids occurring after the second period of withdrawal and the decreased number of amino acids (in the C/I/C regimen) with increased concentrations 24 hours after insulin withdrawal obscured any insulin effects. The longer period of hyperamino-acidaemia in the I/C/I regime might have been caused by supressed endogenous secretion during the initial insulin infusion. In the C/I/C group either insulin secretion or end organ responses could have been primed during the first control period of amino acid infusion, greatest increases being in those infants (An and Ad) not previously infused with Vamin.

The behaviour of tyrosine should be noted: it was the amino acid most resistant to any decrease in concentration after infusion of insulin and the amino acid most often increased after withdrawal of insulin. This may be caused by the late maturation of the p-hydroxyphenyl pyruvic acid oxidase enzyme complex in the liver of preterm infants being superimposed on the changes in concentration of amino acids occurring in response to altered insulin concentrations.

Essential and non essential amino acids appeared to respond to insulin concentrations in a similar manner. Changes in the ratio used by Whitehead[43] - total molar concentrations of plasma glycine, serine, glutamine and

taurine: leucine, isoleucine, valine and methionine - which may be regarded as an index of nutritional status (high values being found in cases of protein-calorie malnutrition) were random and showed no significant difference between periods on or off insulin infusion in the same infant. However, after 24 hours of insulin infusion on 10 out of 12 possible occasions the ratio was decreased. The mean and S.D. ratio value for all infants when off insulin was 2.47 ± 0.99 ($n = 28$) and when on insulin 2.15 ± 0.95 ($n = 28$).

Infusion of insulin with amino acids during the first 24 hour trial period usually resulted in either a decreased or unchanged plasma amino acid concentrations within the first 3 hours, whereas infusion of amino acids alone, whether previously infused or not, always resulted in increased plasma amino acid concentrations. Thus although no significant effect was noted, insulin infusion abolished the increase in amino acids which normally occurred with the start of amino acid infusion.

The exception to this was Gr, an infant of a diabetic mother, in whom amino acid concentrations increased at 3 hours post insulin even although amino acid infusion had commenced 3 days previously. The effect of maternal diabetes on fetal plasma glucose and insulin concentrations *in utero* might have altered the infant's usual postnatal response, insulin release normally being depressed during the first few days of life. Increased endogenous insulin secretion from β islet cell hyperplasia could result in a smaller net increment of insulin on infusion. In addition

end organ response might be more effective and glucagon release and change to lipid metabolism swifter - blood glucose concentration remaining relatively constant (N.B. the study commenced on day 5 and therefore beyond the normal postnatal period of hypoglycaemia of infants of diabetic mothers.)

Examining changes in individual infants, twenty four hours after commencing the insulin and amino acid infusion in schedule 2 plasma amino acid concentrations increased above the initial concentrations in two infants (Ad and B1) but in another decreased to concentrations below those of the first sample - although it should be noted the concentrations in this infant - Ga - were high in the original sample. In An and Gr the concentrations of amino acids were similar to the initial concentration, some amino acids being slightly increased and some slightly decreased. The opposite trend occurred in the infants initially infused with amino acids alone, plasma amino acid concentrations increasing at 3 hours and then falling during the subsequent 21 hours. As amino acid infusion was commenced at the same time as insulin for An and Ad and within 12 hours of intravenous feeding for Gr the observed reduction of plasma amino acids may not be a total reflection of insulin's effectiveness in lowering plasma amino acid concentrations. In infants receiving a second period of insulin infusion (An, Ad and B1) the decrease in amino acid concentrations after 3 hours was observed only in the plasma of Ad, plasma amino acid concentrations

in B1 and An being increased. Although the absolute decrease was greater in the second period of insulin infusion in Ad, the percentage decrease was greater during the initial period. After 24 hours the plasma amino acid concentrations of Ad was further decreased, and although in both An and B1 the plasma amino acid concentration was below that of the 3 hour post insulin sample only in An were plasma amino acid concentrations consistently decreased compared with the sample taken before commencing insulin.

The high concentrations of amino acid found in Ad's plasma during amino acid infusion might be a reflection of a catabolic state. The decrease in amino acids during insulin infusion and the increase during the second 24 hours when off insulin would suggest that exogenous insulin was capable of stimulating cellular uptake. Unknown factors are the rate of protein synthesis as opposed to increased cellular amino acid content, and the effect of catabolic hormones such as hydrocortizone and nonadrenaline in conditions of stress.

The continued low plasma amino acid concentrations in B1 during amino acid infusion both with and without insulin suggests that amino acids were being utilised for synthesis and weight gain would confirm this (1.44 to 1.55 kg in 4 days). This could explain the absence of an increase in amino acids when insulin infusion stopped at the end of the first 24 hour period. Prolonged infusion of Vamin prior to this might have altered responses or conceivably catabolic hormones release might have been low

in the anabolic state which had been achieved. Also, it is possible that a greater release of endogenous insulin could have resulted in a reduced response to exogenous insulin during the second 24 hour infusion period.

The three infants on regimen (1) - off/on/off insulin all showed an increase in plasma free amino acid concentrations at 3 hours but then subsequently decreased towards pre insulin values. Stimulation of endogenous insulin secretion with resultant increased cellular uptake could explain this pattern of changing concentrations. Increased protein synthesis is also indicated as even during the second period off insulin amino acids remained within the normal range. Insulin during the second 24 hour period in most instances reduced the concentration of amino acids in the first 3 hours of insulin infusion and insulin further decreased it during the next 21 hours. This suggests that cellular amino acid uptake was not maximally stimulated by the endogenous insulin secretion during the first 24 hour period. Although in Fo a "rebound" increase (approximately doubling of the concentration) was noted on terminating insulin infusion, twenty four hours later the concentration had returned to that of the original, 24 and 48 hour samples - i.e. there appeared to be a rapid adaptation to either increased or decreased insulin concentrations. Plasma amino acids in Tw were observed to increase 3 hours after withdrawing insulin, but unlike Fo, who had previously been infused with amino acids, they continued to increase to concentrations greater than those found after the initial infusion without insulin.

Thus, simultaneous administration of insulin when a

solution of crystalline amino acid is first infused appears to prevent immediate hyperaminoacidaemia. After a short period of time adaptation by the infant to this new level of insulin and the basic metabolic state of the infant e.g. anabolic or catabolic, may counteract effects of exogenous insulin and be more important in determining plasma amino acid concentrations. This would imply that protein synthesis is stimulated by an initial increase in the concentration of the anabolic hormone insulin, but that prolonged stimulation depends on other factors e.g. energy supply. There is a complex interaction between energy supply amino acids and insulin involving inter alia, glucagon, hydrocortisone, noradrenaline and the acid-base status of the infant.

Reduced concentrations of plasma amino acids observed during the experimental period were not a result of renal overflow. Amino acid analysis of 24 hour urine samples corresponding to the 24 hour periods on and off insulin did not reveal any significant or consistent changes with a change in regimen. Even when very high plasma concentrations of amino acids were recorded (Ga and Ad) no renal overflow was observed. Glycine and proline were the only amino acids consistently excreted in large quantities - whether on or off insulin - this being in accord with the known immaturity of the glycine and amino acid transport mechanism within the kidney tubule during the first weeks of life (see p. 15). The high concentrations of amino acids in the urine Gt represent urinary concentration - total urine volumes for the two 24 hour periods being only 40 and 31 mls. with

osmolality values of 460 and 485 mosm/kg respectively. Urine output and osmolality are within the ranges 31-270 mls and 135-485 mosm/kg respectively. No correlation between volume and the osmolality of urine existed, 31.9 ± 19.0 mosmoles being the mean daily excretions of the whole group. However, individual variation, apart from Fo on day 2 is smaller.

Plasma osmolality was particularly measured because of the very high osmolality - approx 1400 mosm/kg of Vamin solution. With the exception of Ad in whom plasma osmolality of 333 mosm/kg at 48 hours and 326 mosm/kg at 72 hours and An with plasma osmolality of 330 at 48 hours were recorded, osmolality values in plasma ranged between 278 and 308 mosm/kg. Zero hour samples ranged between 282 and 295 mosm/kg. During infusion of amino acids osmolalities increased within 3 hours in all but Ad's plasma, but no consistent pattern with changing insulin regimens were subsequently found. Again with the exception of Ad and An osmolality changes between any 2 samples were less than 15 mosm/kg and usually below 10 mosm/kg, but Ad and An showed marked "swings" on changing insulin regimens with a low of 295 mosm/kg and a high of 333 mosm/kg.

Conclusions

Although the number of infants studied was small, an indication of the effect of altered insulin concentrations on plasma amino acid concentration can be found in the data.

Apart from the expected lowering of blood glucose concentration, infusion of insulin lowers the concentration

of plasma free L-amino acids without increasing urine excretion. Therefore by inference insulin encourages entry of amino acids into cells thereby encouraging protein synthesis - page 45 However, the effect of insulin may not be sustained, amino acid concentrations returning towards pre-insulin concentration indicating adaptation to the higher insulin concentration. Adaptation could be a result of altered sensitivity of the end organ response, diminished endogenous secretion or a shortened insulin half-life, or increased secretion of glucagon,nor adrenaline or corticosteroids. Hyperamino-acidaemia often occurs on insulin withdrawal but this also may be of a transient nature, possibly depending on the endogenous insulin secretion or end organ response - which may be increased if previously exposed to high amino acid infusion rates while receiving exogenous insulin.

Further study should include a larger number of infants in these schedules and a number of infants in whom the infusion periods are extended - perhaps to 3 days. Additionally the effect on plasma amino acids of giving a single bolus of insulin - given in preterm infants if glucose concentrations are greater than 130 mg/100 ml - should be examined.

CONCLUSIONS

Placental transfer of amino acids from mother to fetus is rapid. Subsequently, the transferred free amino acids are quickly cleared from fetal plasma, only a small proportion of the infused amino acid being present in plasma water. Under the experimental conditions employed, plasma free amino acid S.A.reached a plateau 2 hours after a bolus injection given at the start of the continuous infusion of labelled amino acid. This was in contrast to total plasma activity, in which there was a linear increase throughout the infusion. This indicated that rapid attachment to or incorporation into and release of fetal plasma proteins, particularly albumin, must occur. It was proved that the labelled amino acids were incorporated into proteins and that this incorporation and release was approximately 2.32 (phenylalanine) to 7.95 (lysine) μmol/hr/g plasma protein. Tissues taken at termination of the experiments also showed extensive incorporation of labelled amino acids into tissue protein. Thus a slow transfer of amino acids e.g. because of placental malfunction or maternal circulatory problems could rapidly reduce the protein synthetic rate. This should be considered not only from the clinical viewpoint but also in the design of experiments, any change in the distribution or rate of maternal blood circulation from that in the physiological normal state probably having a marked influence on the estimated rates of protein synthesis.

Some protection from short term fluctuations in the supply of amino acids to the fetus may be given by the placenta

It may act as a reservoir whereby amino acids from maternal blood are accumulated and then released only slowly to the fetus. This would allow a continued release of amino acids to the fetus at times when blood circulation through the placenta or maternal plasma amino acid concentrations were temporarily reduced. Evidence for this reservoir role was seen in the different patterns of increase in fetal plasma total activity after ending maternal and fetal infusions. Whereas there was an abrupt halt to the increase in fetal plasma total activity after fetal infusions, fetal activity continued to increase after the infusion into mother had ceased. Calculation of placental transfer rates based on the fetal plasma flux of leucine, lysine and phenylalanine (i.e. their clearance rates from fetal plasma) gave values of + 0.213 (phenylalanine) + 0.073 (leucine) and + 0.060 (lysine) μg N_2/kg/day, values similar to those obtained by measurement of uv:ua concentration differences and umbilical blood flow in unstressed fetal lambs of similar gestation[124]. The effect of insulin on these transfer rates was variable. Although the plasma concentrations of leucine, lysine and phenylalanine all decreased, the S.A. of lysine and phenylalanine increased whereas that of leucine was unchanged during the first hour of insulin infusion.

These observations suggest that, as previously found in <u>in vitro</u> and <u>in vivo</u> acute experiments, insulin removes amino acids from the extracellular to the intracellular compartment. The increase in S.A. would imply that the placenta is initially incapable of accelerating transfer

rates sufficiently to compensate for this removal, or may itself remove amino acids from fetal plasma. However the return of amino acid S.A. to pre-insulin values during continuous insulin infusion would suggest there was only a temporary effect of insulin on placental transfer rates, and that the fetus adapts to a higher basal insulin concentration. The unaltered leucine S.A. would indicate that either insulin influences individual amino acid transfer rates or that there is individual variation in the response to insulin. Variation in the response to insulin by different tissues, particularly liver and muscle, could alter the change in S.A. of amino acids such as leucine, which is primarily taken up by muscle.

The rapid change in phenylalanine and lysine S.A. on infusing insulin, followed by a slow return towards pre-insulin values could suggest that fluctuations are of greater importance than the absolute insulin concentration in determining the metabolic response to insulin. The unaltered plasma total activity, and unchanged or decreased rate of ^{14}C incorporation per gram of protein released into plasma during insulin infusion supports the concept that insulin promotes growth i.e. net protein synthesis by decreasing catabolism rather than increasing the rate of protein synthesis. Thus if the placental transfer of glucose (or gluconeogenic amino acids such as alanine and glutamine) were increased sufficiently to satisfy energy requirements, increased synthesis could temporarily occur at the same amino acid placental transfer rate. A sustained increase in net synthesis would, however, eventually require an increased transfer of amino acids from

mother to fetus. Fluctuations in supply, allowing short periods of a higher rate of net synthesis might result in a faster overall growth rate if these were followed by a brief period of increased placental transfer to replace the incorporated amino acids. Thus in diabetic pregnancies the greater fluctuation of maternal insulin and glucose concentrations may have a more significant influence on fetal metabolism and growth than their actual concentrations. Prolonged maternal hyperglycaemia and fetal hyperinsulinism might by increasing placental size and surface area increase nett amino acid transfer/protein synthesis. /and

In preterm parenterally fed infants increasing plasma insulin concentration by infusion of insulin at 0.1 or 0.2 iu/kg/hr for a period of 24 hours also produced a variable metabolic response. Although plasma amino acid concentrations decreased, both the percentage and absolute decrease varied and was for and after a variable time. Discontinuation of insulin infusion increased plasma amino acid concentrations, but the timing and degree of the response was also variable. The clinical and metabolic status of the individual infant, postnatal age and duration of amino acid infusion prior to insulin administration were of more significance in determining the absolute plasma amino acid concentration, although insulin did affect the plasma amino acid concentrations of both anabolic and catabolic infants, infants of three weeks post natal age receiving parenteral nutrition from birth and neonates previously fed only dextrose. Continuous infusion of insulin may therefore promote protein synthesis in "healthy" preterm infants but in a "sick" catabolic infant, insulin may only cause a dangerous increase in the intracellular amino

acid concentration, (particularly if amino acids are already at high concentrations) without promoting the utilisation of amino acids in protein synthesis. Prolonged insulin therapy may also result in supressed endogenous insulin release, adaptation of end organ response to the higher insulin concentration and alteration in other antagonistic hormonal responses e.g. glucagon and catecholamines. Thus there may be little long term benefit from continuous insulin infusion. Additionally, sudden withdrawal of insulin, from either an anabolic or catabolic infant could result in a period of hyperaminoacidaemia, and if insulin supresses catabolism, in increased catabolism. Insulin infusion in the newborn human infant therefore needs careful biochemical monitoring of not only plasma glucose, but also amino acids, if any advantages are to be gained.

APPENDIX

APPENDIX 1

Biuret Reagent

0.15 g reagent copper sulphate $CuSO_4.5H_2O$ and 0.60 g reagent grade sodium-potassium tartrate $COOK_{\circ}CHOH.CHOH.COONa.-4H_2O$ were dissolved in 50 ml water and transferred to 100 ml volumetric flask. 30 ml 10 per cent (2.5 N) carbonate-free sodium hydroxide NaOH was added with stirring and the reagent made up to the mark with distilled water.

APPENDIX 2

Non Esterifield Fatty Acid Reagent

Solution A: Cobalt nitrate-acetic acid - potassium sulphate was prepared by adding 6 g $Co(NO_3)_26H_2O$ and 0.8 ml glacial acetic acid to a filtered saturated solution of K_2SO_4 - additional potassium sulphate solution being added to 100 ml.

Solution B: Sodium sulphate Na_2SO_4 was added to boiling water until saturated.

Cobalt Reagent

1.35 ml triethanolamine, 8.65 ml Solution A and 7 ml Solution B were well mixed together. The reagent is unstable therefore was freshly prepared for each batch of analyses.

APPENDIX 3

Phosphate Buffer

10 mmol/l sodium phosphate

150 mmol/l sodium chloride

15 mmol/l sodium azide

APPENDIX 4

Amino Acid Buffers

Buffer 1	-	pH 2.85	-	46.5 g Lithium citrate
				100 ml Lithium hydroxide
				40 ml Brij
				20 ml Thiodiglycol
Buffer 2	-	pH 3.75	-	28.2 g Lithium citrate
				50 ml Lithium hydroxide
				20 ml Brij
				10 ml Thiodiglycol
Buffer 3	-	pH 6.25	-	46.4 Lithium citrate
				168.4 g Lithium chloride
				100 ml Lithium hydroxide
				40 ml Brij

Buffers 1 and 3 were made up to 4 litres and buffer 2 to 2 litres with cooled boiled deionized distilled water, titrated with 6N HCl to the required pH and filtered through Whatman GF filter paper. A little phenol was added to each as a preservative.

APPENDIX 5

Ninhydrin Reagent

20 g	Ninhydrin (Koch-Light) for amino acid analysis
3 g	Hydrindantin
3000 ml	2-methoxyethanol (methyl-cellosolve)
400 ml	Sodium acetate buffer
600 ml	Cooled boiled distilled deionized water

Ninhydrin and hydrindantin were dissolved in methyl cellosolve before adding the sodium acetate buffer and water. The deep red solution was filtered through Whatman G.F. papers before bubbling with oxygen free nitrogen for 30 minutes to displace the air. The ninhydrin reagent was stored under nitrogen.

Sodium Acetate Buffer

1088 g Sodium acetate trihydrate

200 ml A.R. glacial acetic acid

Made up to 2 litres with distilled water.

Hydrindantin

80 g Ninhydrin

80 g Ascorbic acid

Ascorbic acid in approximately 100 ml distilled water at 40°C was added to ninhydrin in 2 litres of water at 90°C. Gentle heating was continued for 15 minutes and then the solution allowed to cool slowly. The precipitated hydrindantin was filtered off using a Buchner funnel and the precipitate dried for 3 nights in a vaccum dessicator over phosphorus pentoxide.

APPENDIX 6

Haematocrit Values of Maternal and Fetal Samples

		C1		C2		C3		C4	
		Mat	Fet	Mat	Fet	Mat	Fet	Mat	Fet
Hours	0	26	35.5	28.5	35	30	28	28	36
	15							26.5	35
	30	25	35.5	29	36.5	29	27	27	35
	1	26	35.5	28	36.5	30	27	26.5	35
	1½	25	-	28	-	30.5	-	27.5	-
	2	25	35	28.5	36	30	27.5	27.5	35.5
	2½	25	-	28.5	-	30	-	27.5	-
	3	26	36	28.5	36.5	30	26	29	37
	3½	25.5	-	29	-	29	-	28.5	-
	4	25	36.5	28.5	36.5	29	25	28.5	36
	4¼								
	4½								
	5	25	35	28	37	28	27.5	29	35
	6	25	35	28	37	27	25	30	34.5
	7								
	7¼								
	7½								
	8	26		29		28		28	
	9								
	10			29	35.5			27.5	31.5
	11	26	34			29	25		

APPENDIX 6 cont

		Ins C1		Ins C2		Ins C3		Ins C4		Ins C5	
		Mat	Fet	Mat	Fet	Mat	Fet	Mat	Fet	Mat	Fet
Hours	0	42.5	41.5	31.0	48.5	31	40	34.5	37	28.5	42.5
	¼		41		48.5		39.5		35		42
	½		41				39.5		35.5		42
	1	40.5	41	29.5	49.0	31	39.5	37	35.5	28	42
	1½										
	2	35.5	40.5	27.5	43.0	30	42.5	30	35.5	28	41.5
	2½										
	3	32.5	41	29.0	42.5	31	41	33	36	28	42.5
	3½										
	4	31.0	40	30.5	44.0	30	40	32	35	28	45
	4¼		38.5		54.0		39		35.5		45
	4½		39						36		42.5
	5	33.0	38.5	29.5	40.0	30	39	31	34	28	44
	6	33.5	37.5	30.0	38.5	31	40	31	34.5	28	44
	7	30.0	37	29.0	52.0	31	40	30	34.5	29	42.5
	7¼		37		45.5		37.5		34.5		42.5
	7½		37				39		35		41
	8	32.0	35.5	27.0	36.0	28	39	30	35	29	41.5
	9	33.0	34	26.0	33.0	29	38.5	30	35	28.5	41
	10			27.0	32.5	30	38.5	30	34	28.5	41
	11										

APPENDIX 6

POST INFUSION HAEMATOCRIT VALUES

		C1		C2		C3		C4		
		M	F	M	F	M	F	M	F	
Day	1	25	34	28	35	-	25	25.5	73	(rptd)
	2	27	33	27	-	27	23	26.5	31	
	3	26	35	26	-	26	25	23.0	28.5	
	4	23	34.5	24.5	-	28.5	24	-	-	
	5	24.5	35	29	-	28	26	26	31	
	6	24	33.5	clotted	-	28	24.5	26	31	
	7	26	37	25	44	35	28	26.5	33.5	
	8	27	-65	-	-	30.5	-	29	34	
	9	27.5	32	-	-	-	-	-	-	

rptd = repeated

APPENDIX 6

Changes in fetal haematocrit percentage values during infusion

Experiment	Zero hour	Final 10 or 11 hours	Maximum at (hour) Max. (time)	Minimum at (hour) Min. (time)	Greatest decrease* and (time (hr) of occurrence)
C1	35.5	34	36.5 (4)	34 (11)	
C2	35	35.5	37 (5)	35.5 (11)	
C3	28	25	28	25 (6)	
C4	36	31.5	37 (3)	31.5 (11)	34.5 to 31.5 (6 to 11)
Ins C1	41.5	34.5	41.5 (0)	34 (11)	45.0 to 42.5 (4¼ - 4½)
Ins C2	48.5	32.5	54 (4½)	32.5	49.0 to 43.0 (1 to 2)
Ins C3	40	38.5	42.5 (2)	37.5 (7¼)	45.5 to 36.0 (7¼ to 8)
Ins C4	37	34	37 (0)	34 (11)	37.0 to 35.0 (0 - ¼ hr)
Ins C5	42.5	41	44 (5)	41 (7)	40.0 to 37.5 (7 to 7¼)

* Change greater than 2 per cent

Rate of increase d/min/ml/hr and correlation coefficient (r)

of rate of increase with time during infusions

Exp	Time Hr	Blood (Total) Mat	r	Fet	r	Plasma (Total) Mat	r	Fet	r	Plasma Deproteinised Mat	r	Fet	r
T2	2 - 4					3.01	0.999						
T3	2 - 4					0.56	0.960	-0.28+	0.987				
T4	½ - 4					3.02	0.999	1.01	0.998				
C1	2 - 4	3.83	0.995	1.92	0.999	5.30	0.952	1.94++	1.000				
	4 - 6	1.00	0.993	0.60	0.933	1.35	0.987	0.58	0.997				
C2	0 - 4	2.18		1.13		3.34	0.997	1.51	0.999				
	4 - 6	0.85		1.00		1.60	0.989	1.13					
C3	0 - 4			24.10	0.978	0.16	0.988	33.79	0.999				
	4 - 6							0.50	0.866				
C4 ^{14}C	½ - 3					3.62	0.999						
	½ - 4							1.35	0.998				
^{3}H													

* 0 - 10 hrs
** 4 - 7 hrs
*** 7 - 10 hrs

\+ 2 - 7 days
++ 3 - 4 hrs
+++ 7 - 7½ hrs

Rate of increase d/min/ml/hr and correlation coefficient (r)

of rate of increase with time during infusions

Exp	Time Hr	Blood (Total) Mat	r	Fet	r	Plasma (Total) Mat	r	Fet	r	Plasma Deproteinised Mat	r	Fet	r
Ins C1	0 - 4	2.45	0.0976			2.92	0.999	0.96	0.997			0.85	0.993
	4 - 6					5.21	0.996						
	4 - 7	2.31	0.993			4.08	0.975	1.43	0.997				
	7 - 9	8.94	0.975			0.38	0.999	1.51	0.983			1.56	0.983
Ins C2	0 - 4	*0.37	0.993	15.52	0.978	1.15	0.978	28.00	0.999				
	4½ - 7					3.13	0.986	22.80	0.993				
	7 - 10					0.29	0.996	20.80	0.991				
Ins C3	0 - 4			13.02	0.998	0.38	0.991	15.70	0.990			4.64	0.995
	4 - 7			14.13	0.996			12.00	0.995				
	0 - 10					0.42	0.998	32.00+++	0.997				
Ins C4	0 - 4	0.08	0.831	10.13	0.998	0.90	0.994	12.61	0.995			2.50	0.997
	4 - 7	0.09	0.945	10.00	0.999	0.62	0.977	14.05	0.994			1.99	0.782
	7 - 10	0.03	0.574	12.97	0.999	0.99	0.920	12.86	0.989			4.06	0.971
Ins C5	0 - 4			0.80	0.959			0.77	0.961			0.57	0.951
	0 - 7			1.89**	0.956			1.18	0.948			1.91	0.971
	0 - 10			1.55***	0.954			1.40	0.978			0.56	0.983

* 0 - 10 hrs
** 4 - 7 hrs
*** 7 - 10 hrs

\+ 2 - 7 days
++ 3 - 4 hrs
+++ 7 - 7½ hrs

APPENDIX 8 cont'd

Rate of increase d/min/ml/hr and correlation coefficient (r)

of rate of increase with time during infusions

Exp	Time Hr	Maximum Plasma total Activity d/min/ml		Free Amino Acid SA * d/ml/nmol	
		Mat	Fet	Mat	Fet
T1	2 - 4	13.50			
T2	2 - 4	6.39	*116		
T3	½ - 4	13.37	5.88		
C1	2 - 4	17.00	9.80	27.5	9.8
C3	0 - 4	17.08	8.00		
C2	0 - 4	1.00	150		193
C4 ^{14}C	½ - 3	15.00	6.40	26.0	7.3
	½ - 4	6.44	130		120
Ins C4	0 - 4	32.20	10.75	30	
Ins C1	0 - 4	1.8	214		225
Ins C2	0 - 4	0.48	131		84
Ins C5	0 - 4	0.91	136		72
Ins C3	0 - 4		13.60		

APPENDIX 9

^{14}C Amino Acid Infusions - Total Protein g/l Plasma

		Experiment							
		C1	C2	C3	C4	Ins C1	Ins C2	Ins C3	Ins C4	Ins C5
Maternal	Sample Time (hr)									
	0		44.8		52.0	60.3	64.2		62.3	
	½	50.9								
	1					62.0				
	2	46.9	57.8		57.3	60.3	65.0		51.9	
	3					60.0			51.0	
	4	42.6	60.9		63.2	58.2	65.5	61.9	53.3	
	4¼					53.8			53.8	
	4½					60.3				
	5					61.5	65.0		58.8	
	6					59.7				
	7					56.8	70.6	62.9	51.9	
	7¼					61.9				
	7½					60.3			51.0	
	8					59.7	65.9		57.1	
	9						64.4			
Ins C1-5	10 or End		62.2			57.6	57.5		57.2	59.9
	22	43.4								

APPENDIX 9 continued

^{14}C Amino Acid Infusions - Total Protein g/l Plasma

		Experiment							
		C1	C2	C3	C4	Ins C1	Ins C2	Ins C3	Ins C4	Ins C5
Fetal	Sample Time (hr)									
	0		34.9	48.4		36.1	23.5			28.6
	1						30.5	26.3	30.5	
	2			42.5		29.8	37.5	29.9	31.1	36.7
	3						36.2	30.5	27.5	
	4					32.7	27.2	29.4	22.2	
	4¼						27.2	29.4	22.0	
	4½									
	5	26.7		39.8	20.8	39.3	22.7	33.6	24.6	32.1
	6		33.1				24.9	38.8	28.8	
	7					36.0	22.7	32.5	28.9	
	7¼						27.5	32.8	30.1	
	7½									
	8					32.5	30.0	32.5	32.1	
	9		31.7				31.6	33.6	36.3	
Ins C1-5	10 or End		33.2		23.4		24.9		36.3	27.7
	11	21.1								

APPENDIX 10

Incorporation Rate of Lysine into Maternal

Plasma Protein

Maternal Infusion	Sample Time	Incorporated Activity d x 10^3/min/g	Free Amino Acid SA d/min/nmol	Total Protein g/l	Amino Acid Incorporation Rate µmol/g protein/hr
Ins C1	1			62.0	
	2	3.16	33.6	60.3	1.56
	3	2.74	22.2	60.0	2.06
	4	2.54	32.0	58.2	1.36
	4¼	4.14	30.8	53.8	2.50
	5	5.39	54.7	61.5	1.60
	6	5.51	32.2	59.7	2.86
	7	0.27	46.0	56.8	0.09
	7¼	6.57	24.2	61.9	4.38
	8	3.58	28.4	59.7	2.11

Incorporated Activity (IA)/hr = (Total Activity at xhr - Free amino acid activity at xhr) - (Total Activity at x-1 hr - Free amino acid activity at x - hr)

$$\text{Amino Acid Incorporation Rate} = \frac{\text{IA}}{\text{SA free amino acid}} \times \frac{1}{\text{protein concentration}}$$

* Calculated using free phenylalanine and tyrosine activities.

APPENDIX 10

Incorporation Rate of Phenylalanine Leucine

and Lysine into Fetal Plasma Protein

Fetal Infusion Sample	Time (hr)	Incorporated Activity d x 10^3/min/ml/hr	Free Amino Acid SA d/min/nmol	Total Protein g/l	Amino Acid Incorporation Rate µmol/g protein/hr
InsC2*	1	15.89	182	30.5	2.86
	2	24.66	218	37.5	3.02
	3	29.15	233	36.2	3.46
	4	9.80	155	27.2	2.32
	5	21.46	272	22.7	3.48
	6	24.18	256	24.9	3.78
	7	15.02	254	22.7	2.61
	7¼	39.52	242	27.5	5.94
	8	23.12	233	30.0	3.31
	9	23.58	220	31.6	3.39
InsC3	1	10.00	80	26.3	4.75
	2	18.24	86	29.9	7.08
	3	17.25	82	30.5	6.90
	4	13.34	94	29.4	4.83
	4¼	8.40	74	29.4	3.86
	5	8.97	80	33.6	3.34
	6	15.47	86	38.8	4.64
	7	10.22	82	32.5	3.83
	7¼	30.04	80	32.8	11.44

	8	17.23	96	32.5	5.52
	9	11.33	90	33.6	4.20
InsC4	1	15.04	62	30.5	7.95
	2	14.84	72	31.1	6.63
	3	13.55	72	27.5	6.84
	4	8.97	70	22.2	5.77
	4¼	9.10	126	22.0	3.28
	5	6.20	180	24.6	1.38
	6	15.48	155	28.8	3.47
	7	10.77	110	28.9	3.38
	7¼	9.60	145	30.1	2.20
	8	13.19	131	32.1	3.14
	9	15.66	64	36.3	6.74

APPENDIX II

Calculations

Correction factors - quenching (see

- Recovery a) concentration

$$\frac{\text{area sample peak}}{\text{area norleucine peak x norleucine equivalent}}$$

$$\text{Norleucine equivalent} = \frac{\text{area known quantity amino acid}}{\text{area known quantity norleucine}}$$

b) radioactivity - sample counts x $\frac{\text{radioactive standard counts}}{\text{radioactive standard counts added}}$

Flux - or Entry or Clearance Rate

$$Q \text{ (flux)} = q\frac{Si}{SA} \ \mu\text{mol/hour}$$

where q = rate of amino acid infusion (μmol/hour)

Si = Specific activity infused amino acid

SA = Plateau Specific Activity

Fractional synthesis rate or turnover rate - Garlick 1969

Soltesz et al 1973

$$\frac{SB}{Si} = \frac{K_2 t}{1-e} - k2t - \frac{K_2}{K_1}$$

Where SB = SA protein bound amino acid

Si = SA free amino acid

K_1 = fract. rate uptake amino acid into protein

K_2 = fractional rate protein breakdown

t = time of infusion

Assuming pool sizes are constant K_1 K_2 and t is sufficiently long $K_1 q = K_2 Q \times K_1/K_2 = Q/q = R$ - where q & Q correspond to amounts/g of free bound amino acid.

Substituting RK_2 for K_1

$$\frac{SB}{Si} = \frac{K_2 t}{1-e} - RK_2 t - \frac{K_2}{RK_2}$$

$$= \frac{K_2 t}{1-e} - RK_2 t - \frac{1}{R}$$

A plot of $\frac{SB}{Si}$ v K curve gives the half lives $t = \frac{\ln 2}{K}$

Protein bound activity, provided adequate washing and careful hydrolysis are carried out can easily be estimated. However the full specific activity of the precursor is controversial, as the true identity - whether total intracellular, discrete intracellular or even extracellular amino acids of the precursor is not entirely agreed. If true intracellular specific activities are calculated the extracellular fluid volume of tissue has first to be established - usually from the ^{14}C inulin space. Then

$$\text{Intracellular space cpm/ml} = \frac{\text{total cpm in tissue} - \text{cpm/ml medium X Inulin space (or extracellular fluid)}}{\text{total tissue water} - \text{Inulin space}}$$

APPENDIX 12.

Dextrose Mineral Cocktail	Per Cent
Dextrose Anhydrous	9.5
M/1 Na lactate	1.29
NcC1	0.27
K C1	0.018
$CaC1_2$ hexahydrate	0.018
$MgSO_4$ hydrate	0.08
Potassium acid phosphate	0.163

Immediately before injection add 4 ml inj Ca gluconate 10 per cent to produce /100 ml -

Na^+ 5.9 mmol	$C1^-$ 5.02 mmol
K^+ 1.44 "	SO_4 = 0.33 mmol
Ca^{++} 098	H_2PO_4- 1.20 mmol
Mg^{++} 0.33	Lact. 1.29 mmol

Dextrose 38 Cals.

APPENDIX 13

Contents	Vamin 7 per cent g/l	
Aspartic acid	4.05	
Glutamic acid	9.00	
Alanine	3.00	
Arginine	3.30	
Cysteine and Cystine	1.40	
Glycine	2.10	
Histidine	2.40	
Isoleucine	3.90	
Leucine	5.25	
Lysine	3.85	
Methionine	1.90	
Phenylalanine	5.45	
Proline	8.10	
Serine	7.50	
Threonine	3.00	
Tryptophan	1.00	
Tyrosine	0.50	
Valine	4.25	
Total amino acids	69.95	
Nitrogen	9.4	
Energy	650	(2.73 MJ)
Glucose	100	

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Foetal amino acids

. Cockburn and Merete Giles, *Department of Child Life and Health, ersity of Edinburgh and Simpson Memorial Maternity Pavilion, Royal mary, Edinburgh*

Maternal factors

aternal dietary protein is the major source of foetal amino acids when rnal nutritional intake and absorption are adequate. In states of maternal in undernutrition or malnutrition foetal amino acid supplies are maintained at xpense of maternal tissue breakdown. Rates of placental amino acid transfer ot readily measurable in man, but measurements of foetal to maternal ratios of idual amino acids have been used to give an indication of the efficiency of ntal transport systems for amino acids (Kerr & Waisman, 1967). Maternal utrition is associated with increased plasma glycine/valine quotients in er and foetus (Lindblad, Rahimtoola, Said, Haque & Khan, 1969). In pre- ıpsia with retarded foetal growth there is a generalized increase in maternal ıa amino acid concentrations towards non-pregnant values and the foetal to rnal plasma amino acid ratios, particularly of the branched-chain amino acids, ower than normal (Cockburn, Blagden, Michie & Forfar, 1971). Higher rnal plasma branched-chain amino acid concentrations may reflect a release of amino acids from maternal muscle protein.

ıe normal fall found in maternal plasma amino acid concentrations during ıancy may be mediated through changes in circulating hormones. Oestrogen, :sterone, cortisol and insulin influence plasma amino acid concentrations ıeman, Seal & Doe, 1967; Dancis, Money, Springer & Levitz, 1968) and the ı maternal plasma concentrations may, in part, be due to an increased urinary :tion (Zinneman, Johnson & Seal, 1963) or to an increased rate of transfer of o acid into cells of uterus and into placenta and foetus (Bjorenesjo, 1968).

Placental factors

ender & Christensen (1959) postulated that amino acids are transported ss cells against concentration gradients from an active uptake site on one side e membrane to produce intracellular accumulation, while on the other side of membrane simple diffusion down a concentration gradient was thought to r. Such a system may function in the placental syncytiotrophoblast since

Pearse & Sornson (1969) have described high concentrations of amino acid in the human placental parenchyma. Hill & Young (1973) demonstrated that in the guinea pig transfer from placental parenchyma to foetal plasma was blocked when foetal plasma acid concentrations exceeded the free amino acid concentrations of the placental parenchyma.

There is controversy about the role of insulin in the regulation of placental amino acid transfer (Villee, 1953; Battaglia, Meschia, Blechner & Barron, 1961; Freinkel, 1965; Grimaldi, Jung & Mahler, 1966; Szabo & Grimaldi, 1970; Demers, Gabbe, Villee & Greep, 1972). The demonstration of insulin receptors in human and animal placentas, with characteristics similar to those in established insulin target tissues such as fat cells and liver, supports the argument that insulin may play a role in the regulation of placental function (Posner, 1974).

Changes in placental blood flow on maternal and foetal sides will influence transport rates. Gross variations in human maternal plasma concentrations of individual amino acids can disturb foetal amino acid metabolism (Cockburn, Farquhar, Forfar & Robins, 1972), but lesser degrees of imbalance in the ewe do not seem to influence unduly foetal lamb values, and there appear to be group transport systems in the trophoblast similar to those of small intestine and renal tubule (Young & McFadyen, 1973).

Foetal factors

The degree to which the foetus can control his own supply of nutrient material is unknown. In a recent Symposium (Elliott & Knight, 1974) on foetal size the maternal and foetal factors controlling foetal growth were reviewed. From the increased rates of growth of infants of diabetic mothers (Persson, 1974) and severe growth retardation in infants lacking pancreatic islets (Liggins, 1974) it appeared that foetal insulin might be a major foetal growth promoting factor. There is evidence in preterm human infants that infusions of insulin can increase the rates of entry of glucose, potassium and amino acids into tissues (Cockburn, 1976). Conversely in rhesus monkey foetuses starved by interfering with placental blood flow, foetal muscle and other tissue protein breakdown occurs and this catabolic state may be associated with catecholamine, glucagon and cortisol release (Hill, 1974).

A small quantity of amino acids may reach the foetus from swallowed amniotic fluid. At term the human infant swallows approximately 450 ml of amniotic fluid per day (Plentl, 1966). The amino acid composition of amniotic fluid changes throughout pregnancy (Cockburn, Robins & Forfar, 1970; Cockburn, Giles, Robins & Forfar, 1973) and this is only in part related to dilution with increasing volumes of urine. Table 1 shows the fall in total mean amino acid concentrations of maternal (MV) and foetal (UA) plasma water, foetal urine (FU) and amniotic fluid (AF) from mid to late pregnancy. In mid pregnancy FU and AF individual free amino acid concentrations correlate but by term these correlations are lost and the fluids are dissimilar (Table 2).

ble 1. *Total mean amino acid concentrations (μmol/l) in fluids from eight mal human pregnancies between 15 and 20 weeks gestation compared with lues from eight normal human pregnancies terminated electively by Caesarean tion between 39 and 40 weeks gestation.*

lues for maternal vein plasma water (MV), umbilical arterial plasma water (UV), foetal urine J) and amniotic fluid (AF) free amino acid concentrations were obtained by summation of ividual free amino acid concentrations measured by column chromatography.)

	15–20 weeks	Ratio/AF	39–40 weeks	Ratio/AF	% Change in mean amino acid concentration
MV	2127	0·89	1807	1·19	15
UA	4509	1·88	3190	2·10	29
FU	2056	0·86	1459	0·96	29
AF	2393	1	1517	1	37

ble 2. *Correlations of 24 to 29 individual free amino acid concentrations in ternal vein plasma (MV), umbilical artery plasma (UA), amniotic fluid (AF) d foetal urine (FU) obtained from eight normal human pregnancies between and 22 weeks' gestation and eight normal human pregnancies terminated ctively by Caesarean section between 39 and 40 weeks' gestation*

MV:AF (15–20) weeks	MV:AF (39–40) weeks	UA:AF (15–20) weeks	UA:AF (39–40) weeks	FU:AF (15–20) weeks	FU:AF (39–40) weeks
24	26	27	25	29	28
TRY	THR	SER (neg)	—	TAU	VAL
HIS	CYS			VAL	LYS
				MET	1-MeHIS
				CYTH	
				ILEU	
				LEU	
				TYR	
				PHE	
				HIS	
				3-MeHIS	

Ratios of amino acid concentrations between foetal UA plasma and urine licate that the foetal kidney at 15–20 weeks gestation can effectively conserve ino acids, possibly reaching an adult level of competence in this respect ockburn *et al.* 1970).

Perfusion of a gravid human uterus at 18 weeks gestation through the uterine eries for 30 min with blood containing L-[^{3}H]phenylalanine confirmed that after y avid uptake of amino acid in syncytiotrophoblast high foetal plasma ncentrations were quickly achieved but very small quantities reached foetal ne. Autoradiographs demonstrated radioactivity in the proximal tubules but this s absent from the distal and collecting tubules (Robins, Baird, Cockburn, ingston & Smith, 1971).

Placental transfer of phenylalanine in chronically catheterised sheep

Placental transfer and foetal uptake of amino acids have been examined in the catheterised foetal lamb after infusions of unlabelled amino acids (Young & McFadyen, 1973; Young, Soltesz, Noakes, Joyce, McFadyen & Lewis, 1975). Clearance rates for different amino acid groups were calculated and the postoperative changes in amino acid metabolism determined. In virtually all studies of amino acid transfer in man, monkey, sheep and guinea pig, concentrations of individual free amino acids measured in plasma water of mother and foetus have been compared. The same is true of experiments employing labelled amino acids where activity is measured in the supernatant plasma water and the precipitated plasma proteins are discarded. However, when labelled amino acid is infused into animals a greater portion of radioactivity is found to be associated with plasma protein than in plasma water. Fig. 1 shows the proportions

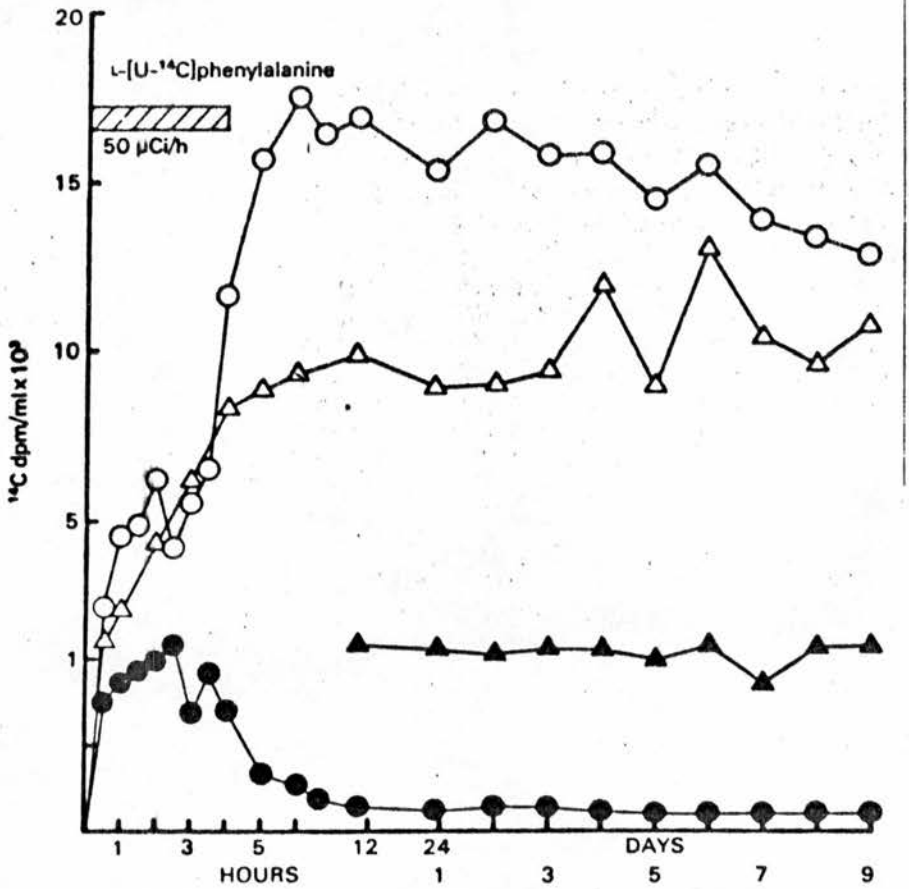

Fig. 1. Relative concentrations of radioactivity in maternal plasma (○), maternal plasma water (●), foetal plasma (△) and foetal plasma water (▲) during and after the infusion of L-[U-^{14}C]phenylalanine into a jugular vein of a 126 d pregnant sheep whose single foetus had foetal UV and UA catheters inserted at 106 d gestation. 200 μCi of uniformly L-[U^{14}C]phenylalanine was infused in 20 ml saline (9 g NaCl/l) at a constant rate of 50 μCi/h for 4 h after a priming injection of 50 μCi in 5 ml saline.

radioactivity found in 1 ml of maternal plasma compared with that found in asma water from 1 ml of deproteinized plasma (plasma–0·6 M-sulphosalicyclic id, 1:1 v/v). Samples were obtained during and after the infusion of L-[U-C]phenylalanine into the jugular vein of a 126 d pregnant sheep whose single etus had foetal UV and UA catheters inserted under general anaesthesia at 106 d estation (see Mellor & Matheson, 1975 for the techniques of catheterisation and re). 200 μCi of [U-^{14}C]phenylalanine was infused in 20 ml saline (9 g NaCl/l) at a nstant rate of 50 μCi/h for 4 h after a priming injection of 50 μCi in 5 ml saline. laternal blood samples were obtained from the other jugular vein and foetal mples from the UA catheter. It is evident that plasma water activity is at a much wer level than that of whole plasma and that whereas plasma water values reach w concentrations after 12 h, high activity is retained in association with protein uring the 9 d of study.

Foetal plasma water activity is higher than maternal and this is in accord with e high foetal to maternal ratios found in other studies; and foetal plasma protein tivity is high in comparison with foetal plasma water, though it does not reach e values of maternal plasma protein. Maternal and foetal plasma protein tivities are converging by the 9th day after infusion. When specific activities are nsidered for phenylalanine in maternal plasma water there is a fall from 79×10^3 m/μmole at 2 h to 1×10^3 dpm/μmole at 22 h. Values remained at about this level uring the next 7 days. In foetal plasma water phenylalanine specific activity was $\times10^3$ dpm/μmole at 5 h, 5×10^3 dpm/μmole at 11 h and $0{\cdot}1\times10^3$ dpm/μmole at d. Maternal plasma water ratios of tyrosine specific activity to phenylalanine ecific activity increased fairly rapidly from 0·008 at 30 min to 0·7 by 22 h.

Acid hydrolysis of the plasma protein sulphosalicylic acid precipitates confirms at 70–110% maternal plasma protein-associated activity in the samples so far alysed is accounted for by tyrosine and phenylalanine.

From the end of the infusion until the end of the experiment phenylalanine tivity averaged 222 dpm/mg plasma protein in maternal plasma and 174 m/mg foetal plasma protein. Mean maternal plasma protein concentration was g/l whereas in the foetus this value was only 25·3 g/l.

Identical patterns of dissociation between plasma water and plasma protein tivities have been found during [^{14}C]leucine infusions into the ewe.

Infusions of [^{14}C]phenylalanine into the foetus demonstrate (a) the very rapid sociation of radioactivity with foetal plasma protein, (b) that foetal plasma water ncentrations exceed maternal and (c) that maternal protein-associated activity ceeds maternal plasma water activity (Fig. 2).

When [^{14}C]phenylalanine or [^{14}C]leucine is added to blood in a test tube or in a nometer maintaining the blood at a 'physiological' temperature, pH, P_{O_2} and $_{CO_2}$ the activity remains in the plasma water fraction. Within 10 min in vivo a rge proportion of radioactive amino acid is protein-associated. Phenylalanine and ucine are firmly attached to or are incorporated into protein or peptides and the ture of this association is currently under investigation.

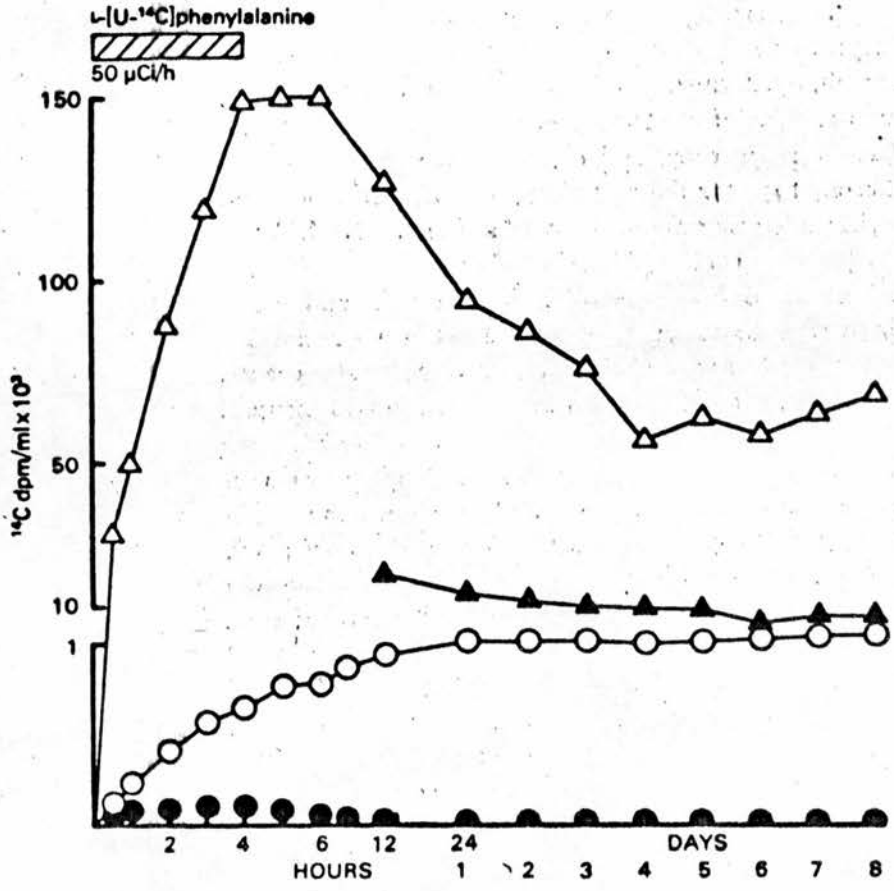

Fig. 2. Relative concentrations of radioactivity in foetal plasma (△), foetal plasma water (▲), maternal plasma (○) and maternal plasma water (●) during and after the infusion of L-[U-^{14}C]phenylalanine into an umbilical vein of a 130 d single foetus which had UV and UA catheters inserted at 110 d gestation. 200 μCi of uniformly labelled L-phenylalanine was infused in 20 ml saline at a constant rate of 50 μCi/h for 4 h after a priming injection of 50 μCi in 5 ml saline.

The role of insulin in placental amino acid transfer

After a meal containing protein or amino acid, maternal plasma amino acid concentrations rise and normally increased insulin secretion will result. This rise in maternal insulin will increase insulin attachment at receptor sites on the maternal aspect of syncytiotrophoblast and encourage the transfer of amino acids into placental parenchyma. From placental parenchyma amino acids could diffuse down a concentration gradient to foetal plasma water and in turn be removed from foetal plasma water to the intracellular compartments of the foetus at a rate mediated by foetal insulin secretion.

Information gathered about foetal amino acid metabolism in different animal species is beginning to shed light on factors which control foetal amino acid

stribution, protein synthesis and growth. More information is necessary before empts can be made to improve the well being of a foetus suffering from rauterine malnutrition.

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